ABSTRACT BOOK

EMBO Workshop

EMBO workshop: Imaging the Immune System

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Lugano, Switzerland

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P1

CD8⁺ T cells require signal 3 from non-antigen-presenting cells for optimal clonal expansion

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Background

CD8⁺ T cells require the integration of three types of signals (antigen, costimulation and cytokines) for their activation. While antigen-presenting dendritic cells (DCs) can provide all three signals required for CD8⁺ T cell activation, it remains elusive whether interactions with DCs suffice for the generation of a high number of effector CD8⁺ T cells.

Methods

To strictly control the number of antigen-presenting DCs and antigen-specific CD8⁺ T cells, we employed a reductionist model whereby recipient mice received 10,000 ovalbumin-specific CD8⁺ T (OT-I) cells one day before subcutaneous immunization with bone marrow-derived DCs (BMDCs) loaded with OT-I peptide. Using light sheet fluorescence microscopy, we determined the ratio of BMDC to OT-I cell in reactive lymph node (LN) during the first days of a burgeoning CD8⁺ T cell response. We then correlated the BMDC/OT-I ratio at the early phase to OT-I cell number 7 days after immunization.

Results

Each antigen-presenting DCs can simultaneously engage with multiple T cells, permitting one DC to act as antigenpresenting cells for more than ten antigen-specific T cells under certain conditions. However, we found that induction of effector OT-I cell response required approximately 8-times higher number of BMDCs than OT-I cells at 24 hr post-immunization. In contrast, OT-I cells successfully generated a high number of effector cells even when a large fraction of BMDCs were replaced with those that are not loaded with OT-I peptide. In the presence of activated yet not antigen-loaded BMDCs, less than one antigen-presenting DC per OT-I cell induced the generation of effector OT-I cells. We found that IL-12 was critical for the action of non-antigen-presenting BMDCs. Interestingly, however, the point of action of IL-12 was bystander immune cells in the reactive LN, but not reactive CD8⁺ T cells.

Conclusions

We found that the number of DCs as antigenpresenting cells can be lower than that of reactive T cells in the reactive LN, as long as other cells, including activated yet non-antigen-presenting DCs, secrete sufficient levels of inflammatory mediators. Our data suggest that CD8⁺ T cells sense the local level of inflammation in the reactive LN and adapt effector cell generation to it. Conceivably, such a prerequisite for effector CD8⁺ T cell responses acts as a safeguard against accidental priming of autoreactive CD8⁺ T cells by a few “rogue” DCs.

Dendritic cell ICAM-1 strengthens immune synapses with CD8 T cells but is dispensable for early T cell differentiation

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Lymphocyte priming in lymph nodes (LNs) depends on the formation of functional TCR specific immune synapses (ISs) with antigen (Ag) presenting dendritic cells. The high affinity LFA-1 ligand ICAM-1 has been implicated in different ISs studied *in vitro*. The *in vivo* roles of DC ICAM-1 in Ag stimulated T cell activation and differentiation have been unclear. In newly generated DC conditional ICAM-1 deficient mice, we found that under Th1 polarizing conditions of either vaccination or viral infection naïve CD8 T cells entering skin draining lymph nodes normally engaged the ICAM-1 deficient Ag presenting DCs and underwent normal priming, proliferation and early differentiation into functional cytotoxic T cells. Single cell RNAseq analysis confirmed that effector and early memory T cells were normally generated in these mice. Notably, however, ICAM-1 deficient DCs could not engage in stable conjugates with newly generated Ag specific CD8 blasts suggesting a key role for DC ICAM-1 in tight synapses generated between subsets of activated CD8 blasts and Ag presenting DCs. Our results suggest that although these CD8 T cell blasts tightly bind DC-ICAM-1, strongly adhesive DC-T ISs are not necessary for functional TCR dependent CD8 T cell proliferation and early differentiation. Thus, differentiation of CD8 lymphocytes can take place without tight LFA-1-ICAM-1 adhesions, probably through serial TCR mediated integrin-independent T cell contacts with Ag presenting DCs. The suggestion that DC ICAM-1 is a critical co-stimulatory molecule in all immune synapses of naïve T cells and Ag presenting DCs must be revisited.

P2

Establishing a mouse model to visualize limitations of CAR-T cells activity in solid tumors

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Background

The use of CAR-T cells (chimeric antigen receptor T cells) has shown promising success in the treatment of B hematological malignancies, but their therapeutic performance in solid tumors remains more limited. Our objective is to decipher the dynamics of CAR-T cells in solid tumors *in vivo* at the single-cell level and to compare it to hematological tumor models to identify limitations to CAR-T cells efficacy.

Methods

For this purpose, we developed a murine subcutaneous solid tumor model to study the effect of CAR-T cells therapy. We generated tumor cell lines expressing a fluorescent probe to monitor apoptosis (the DEVD probe) and expressing the CD19 antigen. Response to CAR-T cells is then analyzed by flow cytometry and intravital imaging.

Results

We found that CAR-T cells therapy in this solid tumor model prolong mouse survival and delay tumor growth but could not induce complete remission. Moreover, we could visualize CAR-T cells infiltration at the tumor site. Therefore, our model is suitable to visualize potential limitations of CAR-T cells therapy. We are currently using intravital two-photon imaging to decipher CAR-T cells behavior and killing potential during the course of CAR-T cells therapy.

Conclusions

In sum, we have established a new model suitable for intravital imaging that will help identify limitations of CAR-T cells activity in the context of a solid tumor.

P3

A vital role of α-actinins for Lck membrane distribution and immunological synapse formation during T-cell activation

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The initiation of T-cell signalling is critically dependent on the function of Lck kinase. We and others have previously demonstrated that TCR triggering requires the activation-induced redistribution of active Lck within the plasma membrane. In our previous research, we identified the transient formation of Lck-RACK1 complexes that appear early after TCR triggering. Nevertheless, this interaction turned out to be rather indirect, suggesting the formation of a larger multiprotein complex involving RACK1 and Lck. Comparative mass spectrometry analysis of RACK1 interactors between non-activated and activated primary mouse T-cells identified potential interaction partners of RACK1, including α-actinin-1 (ACTN1). Upon T-cell activation, the association of RACK1 with ACTN1 exhibited similar kinetics to that of Lck. In addition, this interaction depends on Lck kinase activity suggesting that active Lck is required for the formation of Lck-RACK1-ACTN1 complexes. The 3D-high resolution live cell imaging microscopy then showed, that within less than 1 minute after initial engagement of TCR, all these proteins are synchronously relocated towards the immunological synapse (IS). To gain further insight into the role of α-actinins family in early T-cell activation events, both ACTN1 and ACTN4 genes were deleted using Crispr-Cas9 technology and distribution of fluorescently-tagged Lck was visualized during IS formation by high-resolution spinning disc confocal microscopy. We observed that Lck kinase was arrested at the IS periphery early after the start of IS formation which is indicative of the impairment of Lck redistribution. In control cells, Lck was evenly distributed across the contact site formed by IS. As expected, such spatio-temporal disorganization has functional consequences. Deletion of ACTN1, or both ACTN1 and ACTN4, led to reduced responsiveness of T-cells. Importantly, in contrast to WT cells, the double KO cells formed only a transient contact with antigen presenting cells, indicating that α-actinins contribute to the stability of the forming IS as well, although the mechanism of this action remains unknown. Together, these results describe RACK1 and α-actinins as relevant intracellular signalling components involved in the regulation of T-cell proximal signalling by the virtue of Lck redistribution within plasma membrane via linking CD4-Lck complex to the cytoskeletal network.

P4

Early stages of HIV-1 infection of the colonic mucosa, imaging the virus with an in-situ hybridization platform

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Background

Human immunodeficiency virus 1 (HIV) infects millions of people each year. Mucosal tissues are the primary site of HIV transmission and a major reservoir for viral replication. HIV sexual transmission occurs by crossing epithelial barriers at mucosal surfaces of the genital and anorectal tracts. We showed with an in vitro mucosal model that HIV can attract antigen presenting cells (APCs) to the apical surface of the colonic mucosa. Due to the lack of techniques to assess the distribution of HIV in tissue, there is little evidence about the anatomic localization of virions and their association with immune cells at the early stages of infection in the colonic mucosa. To address this issue, we utilized the RnaScope platform to detect the localization of HIV.

Methods

Mucosal explants were collected from human rectosigmoid or descending colon far from the colorectal cancer lesion. Tissue fragments, cut with a biopsy punch, were either immersed in or treated apically with the transmitted founder CH106 HIV supernatant at 50,000 Tissue Culture Infectious Dose 50 for 3, 6, 12 and/or 24 hours. Tissues were fixed with formalin, included in paraffin, and sliced 4 microns apart. Subsequential slices were processed for in situ hybridization with a Ventana ULTRA system or alternatively stained to detect CD11c and human epithelial antigen (HEA) with a Leica BOND RX high-throughput stainer. A 20ZZ probe was designed to specifically target HIV pCH106.c/2633 on the Gag-Pol region. HIV RNA was visualized with RNAScope by amplifying target-specific signals. Images were acquired with Aperio® AT2 microscope slide scanner and analyzed with QuPath v0.4.3 software.

Results

HIV was detected at 6 and 12 hours of viral incubation. Thereafter, at 24 hours, detection was decreased, probably due to RNA degradation as the control Ubiquitin was decreased as well. HIV localized inside the epithelium and in the lamina propria of fragments immersed in the viral supernatant while it exhibited a different distribution in tissues stimulated in a polarized manner. The density and location of the virus showed a great degree of variation between donors and among different slice sections from the same sample. In both experimental settings CD11c APCs were detected inside the crypts of the epithelium when stimulated with viral supernatant, while in unstimulated tissues distribution of APCs was mainly confined within the mucosa. Regardless of time of incubation or experimental setting HIV did not co-localize with HEA expressing cells.

Conclusions

Experiments that combine classical immunohistochemistry techniques to visualize immune cells and the RNAScope platform to detect HIV in subsequential slides from tissue are extremely useful to investigate the early targets of HIV-1 infection.

P5

Multiplexed immunofluorescence to identify markers in the tumor microenvironment of DLBCL patients predicting response to CAR-T cell therapy

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Background

Chimeric antigen receptor (CAR) T-cell is a highly personalized immunotherapy that has revolutionized the treatment of hematological malignancies such as diffuse large B-cell lymphomas (DLBCL). In this context, anti-CD19 CAR-T cells are generated in vitro by transducing a genetically engineered CAR protein into autologous T cells. These anti-CD19 CAR-T cells can recognize the CD19 tumor antigen and in turn, are able to eliminate tumoral cells following their re-infusion into the patient. Indeed, CAR-T cell therapy has shown great success with remission rates up to 82% in large B-cell lymphomas (Abramson et al., 2020; Neelapu et al., 2017; Schuster et al., 2019).

Typically, half of the patients progressed or relapsed within 6 months following CAR-T therapy (Abramson et al., 2020; Neelapu et al., 2017; Schuster et al., 2019). Therefore, it appears essential to identify predictive factors of durable response or resistance to CAR-T cells treatment.

A key role of the tumor microenvironment (TME) in lymphomagenesis and response to therapy has now been established: while some immune cells, namely T cells and NK cells, exert an antitumoral role (Schuster 2019 et al., Tarte et al., 2017) other populations such as tumor-associated macrophages (Tarte et al., 2017, Li et al., 2019) are associated with protumoral outcomes. However, the influence of the TME on the efficacy of CAR-T cell therapy remains unknown. It has been shown recently that the expression of macrophages related genes and the presence of non-activated or exhausted CD8+ T cells in patients pre-treatment biopsies were associated with non-durable responses to axicabtagene-ciloleucel therapy (Jain et al., 2021, Scholler et al., 2022). Yet, simultaneous detection of different cell populations and studying how these cells interact together has not been achieved.

Methods

Here we aim to characterize the TME on lymph node pre-treatment biopsies from DLBCL patients treated with CAR-T cells. To this end, we have validated a panel of 28 markers for multiplex immunofluorescence (CODEX) of patient biopsies. We aim to analyze the composition of the TME (cell types and phenotypes), its spatial organization and key cellular interactions.

Results

Preliminary results support extensive variability within the composition of the TME in different patients.

Conclusions

Confronting spatial characterization of the TME prior to therapy to clinical responses should help identify factors predicting the outcome of CAR-T cell therapy.

P6

Development of Fluorescent Probes for Multiplexed PAINT-based Super-Resolution of the Immunological Synapse

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Background

The immunological synapse (IS) that forms between a T cell and its target cell consists of a highiy organized bullseye pattem characterized by distinct receptor subsets in each zone. Whiie this coarse-grained structure is aiready known, the nanoscaie distribution of receptors remains uncharacterized. Recently, the functional role of potential receptor clustering on the surface of immune cells has been intensely debated. Single-molecule localization microscopy (SMLM) provides a powerful tool to reveal this nanoscaie architecture and its functional relevance.

Methods

We are currently generating a library of probes applicable for the SMLM technique of PAINT (Point Accumulation In Nanoscaie Topography), and specifically the variant of PAINT called IRIS (Image Reconstruction by Integrating exchangeable Single-molecule localization). IRIS is based on the resolution of freely diffusing fluorophore-labeied probes (e.g. well-characterized protein docking domains or peptides) that transiently bind to their target epitopes on a fixed biological sample. From a recorded movie, isoiated binding events can be individually localized to <20 nm and used to build up the final super-resolution (SR) image. Our library consists of purified RFP-tagged docking domains produced in Expi293 cells as well as synthesized peptides conjugated to organic dyes. Our SR probes are screened based on detectabiiity, binding kinetics and target specificity (IF counterstaining) on a widefieid Setup for SMLM. For efficient probe handling, a microfluidic pump System is empioyed that also enables sequential probe exchange. Successfully screened probes are used to resolve the IS formed between co-cultured superantigen-pulsed Raji B cells and Jurkat T cells residing in a microfluidic chamber.

Results

We present first results of the assessment of the binding kinetics, optimal concentration and specificity for a library of newly synthesized fluorophore-tagged IRIS probes based on interaction sites of proteins that recognize target epitopes at the IS. We also show preliminary SR Images of the IS formed between T cells and B cells.

Conclusions

Serial SR of the IS in fixed immune cells using our library of newly-generated IRIS probes was established.
Our integrated approach provides a powerful strategy for high-content SR of immune cells and beyond.

P7

Impact of genetic polymorphism in PSA gene on prostate cancer tumor immune-profile

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Background

Our previous analysis in a large cohort of 82,591 prostate cancer (PCa) cases and 61,213 matched controls identified a single nucleotide polymorphism, rs17632542, c.536T>C (Thr163 PSA) in the KLK3 gene to be significantly associated with PCa risk and survival. KLK3 codes for PSA, a prostatic secretory protease, which plays a bidirectional role and is involved in both tumour suppression and progression by regulating the bioavailability and cleavage of growth factors and matrix proteins. PSA is shown to have a role in eliciting T-cell responses. Further, generation of KLK3-specific CD8+T cells was actively engaged in the destruction of tumour cells expressing the cognate antigen, thus leading to anti-tumour responses. Accordingly, it was important to determine whether the rs17632542 SNP would affect some of these functional parameters of PSA.

Methods

We established metastatic PCa models by injecting PC3-Luciferase cells stably expressing wild-type (Wt) or Thr163 mutated PSA in NSG mice. Mice tissue sections were stained using Opal staining kits and analysed using the image cytometry platform StrataQuest.

Results

Intracardiac injected PC3-Wt PSA cells in mice led to lower metastasis indicating a protective role for Wt PSA during metastasis. PC3-Thr163PSA cells injected mice exhibited aggressive metastasis to liver, kidneys and bone compared to Wt PSA groups in support of the poor overall survival in patients with the rs17632542 SNP noted above. The dissected mice bone sections which exhibited higher metastasis for the Thr163PSA group showed a reduced neutrophil count compared to higher neutrophil numbers in both vector control and Wt PSA groups. Using multiplex IF for CD4 [T cells], CD8 [cytotoxic T cells], CD19 [B cells], CD45 [all lymphocytes], CD38 [antibody-secreting plasma cells] and CD138 [plasma cells]), we showed the genotype specific difference in recruitment of these cells to the primary prostate cancer tissue.

Conclusions

Our data suggested that the Thr163 PSA variant may have deleterious effects in the metastatic tumour microenvironment than Wt PSA and may elicit a differential immune response.

P8

How to motivate bone marrow to mobilization? Investigating the effectiveness of cobalt protoporphyrin IX (CoPP) as a candidate for drug used in bone marrow mobilization procedures

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Background

It is estimated that more than 50 000 allogeneic hematopoietic stem cell (HSC) transplantations are performed each year, worldwide. Around 70-95% of these procedures use cells obtained from mobilized peripheral blood. The most widely used mobilizing factor for the treatment of neutropenia and for HSC isolation for further transplantation is recombinant granulocyte colony-stimulating factor (G-CSF). Unfortunately, up to 30% of normal HSC donors and 60% of high-risk patients do not respond effectively enough to G-CSF mobilization. Thus, there is a high demand for development of new bone marrow mobilization strategies. One possible tactic is to create drugs that induce endogenous production of G-CSF. We discovered that cobalt protoporphyrin IX (CoPP) increases endogenous G-CSF production and expression of several other cytokines (e.g. MCP-1, IL-6) that play a key role in the mobilization of cells from the bone marrow into the blood. Based on the preliminary results, we designed a preclinical study to assess the effectiveness of *in vivo* bone marrow mobilization by CoPP.

Methods

We performed a series of experiments on C57BL/6J mice where we tested various doses, different dosage regimens and pharmacodynamics of CoPP injections in comparison to standard G-CSF treatment. We used Luminex assay to analyze key cytokines and chemokines and FACS analysis to investigate blood cells and hematopoietic stem and progenitor cells (HSPCs) that are present in the peripheral blood and bone marrow after CoPP treatment.

Results

We discovered that CoPP in the single dose of at least 5mg/kg is enough to stimulate the mobilization of mature granulocytes to the blood. Pharmacodynamics experiment showed that first cell population mobilized to the blood are granulocytes and we can observe elevated level of these cells just few hours after CoPP injection. HSPCs mobilize to the blood later, and we can observe elevated number of these cells around 48 hours after CoPP injection.

Conclusions

We have shown that CoPP could be used as a potential HSPC mobilization agent. Nevertheless, this project is still ongoing and we are planning to conduct more experiments where we will test antimicrobial and antifungal properties of granulocytes mobilized with CoPP and homing efficiency of HSCs mobilized with this compound.

P9

Changes in lymph node microarchitecture during aging is sensed by naïve T cells and alters nuclear envelope composition

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Background

The COVID-19 pandemic has highlighted the important role of age in disease outcome after infection. Weakened immunity in elderly individuals is partly attributed to a deterioration of T cell responses, known as immunosenescence. Here, we investigated age-related changes to the lymph node (LN) microenvironment in which naïve T cells encounter antigen to understand how this contributes to impaired immunity. In particular, we studied the fibroblastic reticular cell (FRC) network, which provides a scaffold for T cell traffic in the LN, creates conduits to transport antigens, and produces cytokines for T cell survival.

Methods

Using confocal microscopy and custom-built machine learning analyses, we quantified properties and composition of the FRC network of LNs from young (8–12 weeks) and old (>75 weeks) mice. We investigated the impact of the changes in FRC network structure on early LN size increase and T cell expansion following endotoxin administration. We measured the mechanical stiffness, a tissue's resistance to deformation, of LN and spleen from both young and aged mice using a nanoindenter. Finally, we used both in vitro and *in vivo* approaches to explore links between tissue stiffness and mechanosensing by naïve T cells.

Results

We found that aged and young LNs differed in the composition and microarchitecture of the FRC network, including increased expression of fibronectin and collagen I, and a greater density of FRC fibers. Early LN swelling during inflammation was also reduced in aged mice, restricting space for T cell proliferation. Importantly, we demonstrated that secondary lymphoid organs of aged mice had substantially (~4 fold) increased stiffness (Young's elastic modulus). Changes in their mechanical environment were sensed by naïve CD4 and CD8 T cells, leading to increased expression of the nuclear lamina component laminA/C, which impacts nuclear rigidity. Young T cells transferred to aged hosts similarly upregulated laminA/C in response to being in a stiffer environment, but not to levels of endogenous old T cells. Interestingly, using in vitro assays we find that young naïve T cells are unable to survive in stiff collagen matrices as opposed to soft matrices, while naïve T cells from aged mice have a lower rate of death in stiff matrices, suggesting they have adapted to stiffer environments.

Conclusions

Our work establishes that changes in LN microarchitecture during aging lead to altered stiffness sensing by naïve T cells, impacting survival and senescence.

P10

Minimally invasive longitudinal intravital imaging of cellular dynamics in intact long bone

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Background

Intravital two-photon microscopy enables deep tissue imaging at high temporospatial resolution in live animals. However, the endosteal bone compartment and underlying bone marrow pose unique challenges to optical imaging as light is absorbed, scattered and dispersed by thick mineralised bone matrix and the adipose-rich bone marrow. Early bone intravital imaging methods exploited gaps in the cranial sutures to bypass the need to penetrate through cortical bone to image the dynamics of osteoclasts and haematopoietic stem cells (HSCs). However, as a non-weight bearing flat bone, the calvarium differs in its origin, structure and function to long bones like the femur or tibia. Critically the function of osteoblasts, osteocytes and osteoclasts differ between these sites. More recently, investigators have developed invasive methods to thin the cortical bone or implant imaging windows to image cellular dynamics in weightbearing long bones.

Methods

Here we provide a step-by-step protocol for the preparation of animals for minimally-invasive, non-destructive longitudinal intravital imaging of the murine tibia. This method involves the use of mixed bone marrow radiation chimeras to unambiguously double-label osteoclasts and osteomorphs. The tibia is exposed by a simple skin incision and an imaging chamber constructed using thermoconductive T-putty.

Results

We demonstrate the use of this protocol by characterising steady-state osteoclast recycling in the long bone. We reveal regional differences in the rate of osteoclast recycling (osteoclastic fission into osteomorphs, and the subsequent fusion of osteomorphs). High rates of recycling were associated with smaller, more rounded osteoclast/osteomorphs.

Conclusions

This protocol provides a minimally invasive and non-destructive method to imaging osteoclast dynamics in the long bone. Imaging sessions up to 12h long can be repeated over multiple timepoints to provide a longitudinal time window into the endosteal and marrow niches. This method can also be applied to study cellular dynamics in bone remodelling, cancer cell life cycle, haematopoiesis and long-lived humoral and cellular immunity.

P11

Understanding lymph node fibroblastic reticular cell-matrix interactions and implications for fibrotic lung disease

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Background

Idiopathic pulmonary fibrosis (IPF) is a fatal disease characterized by the accumulation of myofibroblasts and excessive extracellular matrix. Enlarged lung draining lymph nodes (LNs) in IPF patients correlate with disease severity, but the underlying mechanisms are unclear. Fibroblastic reticular cells (FRCs) maintain the intricate ECM network in LNs that is crucial for adaptive immune response formation. It is unknown 1) whether FRCs respond to rigidity and composition cues from the ECM similar to fibroblasts in other organs and 2) how the FRC-ECM network is altered in IPF and its subsequent effect on the immune response

Methods

To test whether the ECM ligands fibronectin (FN), laminin (LAM), or collagen I dictate FRC-ECM engagement, we performed single-cell force spectroscopy, an atomic force microscope-based method to measure cell-substrate binding force. A single murine FRC was adhered to a cantilever probe and then lowered to contact the ECM protein of interest for 1, 2, or 5 seconds. The detachment force was measured upon retraction to determine the rate of adhesion strengthening. To investigate the effect of ECM composition and rigidity on FRC phenotype, we seeded FRCs on soft (5 kPa) and stiff (20 kPa) polyacrylamide hydrogels covalently conjugated to either FN or laminin for 48 hours and then immunostained for the stiffness-sensitive transcriptional co-activator YAP as well as paxillin to denote focal adhesion size. The nuclear:cytoplasmic intensity ratio (N:C ratio) of YAP immunostaining was quantified using CellProfiler.

Results

Our preliminary results show that FRCs significantly bind LAM substrates more than both FN and collagen substrates ($p < 0.0001$). Plated on rigid LAM substrate, FRC area increased 3.5-fold compared to those plated on softer LAM substrate ($p < 0.0001$). FRCs spread atop FN substrates regardless of substrate rigidity. Similarly, we observed large focal adhesions on both soft and stiff FN substrates, while only on stiff substrates when coated with LAM. YAP N:C ratio was significantly increased in FRCs plated on FN compared to LAM.

Conclusions

These data suggest that FRCs demonstrate stiffness- and substrate-dependent regulation akin to fibroblasts in other organs. Specifically, the FN-rich provisional matrix commonly observed in fibrosis may activate YAP signaling in FRCs, even in the absence of stiffness. Currently, we are investigating ECM-FRC-immune cell interactions in human IPF and control LNs using 30-plex imaging.

P12

Immunomodulatory effects of gene electrotransfer of plasmid DNA encoding chemokines CCL5 and CCL17 in combination with irradiation in murine tumors

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Background

Chemokines regulate immune cell migration. The degree and the type of immune cells in the tumor affects disease progression and correlates with the efficacy and outcome of immunotherapies. Similarly, beneficial immunomodulatory effects were also observed after irradiation. Therefore, we sought to investigate gene electrotransfer (GET) of proinflammatory chemokines CCL5 or CCL17 in combination with irradiation, as a potential therapeutic strategy for cancer therapy.

Methods

Tumor models were chosen to correspond to an inflamed (CT26 murine colon cancer) or immunosuppressive (4T1 murine breast cancer) immunophenotype. First, chemotactic properties of investigated chemokines were examined in vitro. Next, the potential of chemokines to induce the extravasation of fluorescently labelled splenocytes was determined using intravital microscopy of tumors in dorsal window chamber model (DWC). The antitumor effectiveness of combined therapy utilizing GET of chemokines and two irradiation regimes (single dose of 10 Gy and fractionated dose of 3x 5 Gy) was then determined *in vivo*. Lastly, qRT-PCR was used to evaluate gene expression of several cytokines in tumors after the therapies, while changes in the abundance of CD4+, CD8+ cells and vasculature (CD31+ cells) were determined with immunofluorescent staining.

Results

Both chemokines CCL5 and CCL17 induced the migration of murine macrophages RAW264.7 in vitro. Similarly, in both CT26 and 4T1 tumors growing in DWC, GET of chemokines showed increased retention of splenocytes compared to control. CT26 tumor growth delay after combined therapy of GET of chemokines and both irradiation regimes was significantly longer compared to control and led to tumor cures. In the case of 4T1 tumors, only GET of chemokines combined with irradiation led to a pronounced tumor growth delay but without tumor cures. Gene expression analysis showed increased expression of both chemokines after corresponding therapies. Moreover, increased expression of CXCL9 and CXCL10, two potent chemoattractants of cytotoxic CD8+ T lymphocytes, was determined in tumors after most of the combined therapies. Immunofluorescence showed increased numbers of CD4+ and CD8+ T lymphocytes in tumors after GET of chemokines, however their numbers decreased whenever irradiation was used.

Conclusions

Our results show the potential of chemokines in cancer immunotherapy, however additional optimization of combined therapy is needed before translation into clinics.

P13

Assessing the immune microenvironment in glioma models by correlative high field MRI and optical imaging

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Background

Gliomas are malignant brain tumors with an immunosuppressive tumor microenvironment (TME). We have recently implemented the Toll like receptor 7 agonist, CDNP-R848 to efficiently treat preclinical glioma which induces myeloid repolarization and a pronounced proinflammatory shift of the TME[1]. Magnetic resonance imaging (MRI) is the main clinical modality for treatment monitoring of glioma. However, visualizing the immunological key components of the TME is not possible in clinical practice. We hypothesize that light sheet microscopy (LSM) combined with whole brain immunostaining of myeloid and T cells using iDISCO[2] can provide information on immune cell influx, cellular distribution and perturbations that occur after therapy induction and that mediate treatment.

Methods

Preclinical Gl261 glioma were intracranially xenografted and grown for 2 weeks (n=9 mice). After baseline MRI mice were treated with 3 doses of intravenous CDNP-R848 or CDNP vehicle control. Subsequent MRI were performed in week 3 (effector phase) and 4 (clearing phase), followed by correlative tissue clearing (iDISCO for CD3, IBA1 and CD31), LSM and immunohistochemistry.

Results

We have recently shown that CDNP-R848 potently induces regression of established gliomas (ORR: 75%)[1].
This was based on macrophage activation during the effector phase (week 3) after CDNP-R848 treatment.
To examine spatial patterns of immune cells of the TME we investigated cleared mouse brains with immunostained macrophages (Iba1) and T-cells (CD3). Whereas T cell accumulation occurred mainly around peritumoral microvessels, preliminary data showed additional “non-classical” recruitment pathways of myeloid cells with pronounced macrophage accumulation at the ipsilateral choroid plexus, in the corpus collosum and the leptomeninges. Longitudinal assessment of immune cell distribution compared baseline (before treatment), effector phase (week 3, after completion of treatment cycle) and tumor clearing phase (week 4).

Conclusions

LSM offers a novel platform for cellular “ground truth” generation that can be co-registered to MRI datasets. We propose that the recruitment pathways of myeloid and T cells play an important role for the development of response and resistance towards glioma immunotherapy which can be assessed by correlated by MR-LSM.

P14

Metabolic profiling of human intestinal macrophages in native tissue microenvironments

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Background

Macrophages exhibit high plasticity in tissues, as they are able to drastically change their phenotype and function in response to various stimuli. In recent years, immune cell polarization was linked to reprogramming of cellular metabolism, which enables and sustains their respective effector functions. However, tools to assess these immuno-metabolic mechanism in complex tissues are rare and in vitro generated data cannot account for co-founding factors provided by an highly adaptive and tissue-specific microenvironment observed during an immune response. Therefore, spatial and single-cell information on macrophage phenotypes and metabolism is required in order to properly profile and resolve macrophage heterogeneity and their respective metabolic signatures *in vivo*. We are particularly interested in the heterogeneity of human intestinal macrophage/monocyte populations and aim to characterize their respective metabolic-states within native microenvironments.

Methods

Our lab has developed a single-cell multimodal imaging approach for profiling cellular metabolism by combining enzyme- and immunohistochemical staining techniques. We perform activity assays for key enzymes of glycolysis, the PPP and TCA cycle on fresh frozen tissue sections and co-define the respective cellular and structural compartments by multispectral fluorescent imaging. To assess metabolic signatures of human intestinal macrophage in chronic and acute inflammatory states, we made use of surgically obtained biopsies from Crohn's disease patients and from patients suffering acute appendicitis.

Results

Here we present a metabolic imaging approach, which enables us to characterize single cells, their spatial contexts and simultaneously provide key information on their metabolic states or adaptations to an altered microenvironment. We show first results on the metabolic heterogeneity of human intestinal macrophages, considering their tissue localization and inflammatory states in patient-matched material.

Conclusions

Imaging cellular enzyme activities in situ provides a suitable and flexible tool to characterize spatially resolved metabolic signatures of single cells in complex tissue microenvironments. This tool can be used to investigate the impact of an adapting microenvironment on immune cell metabolism and thereby generate a better understanding of immuno-metabolic mechanism, which are likely defining human intestinal macrophages heterogeneity and function *in vivo*.

P15

A Chemokine Signal Prevents Aberrant T Cell Activation By Terminating Their Crosstalk With Dendritic Cells

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CCR7 mediates the colocalization of activated dendritic cells (DCs) and naïve T cells (TN) in the paracortex of lymphoid organs, where its ligands CCL19 and CCL21 are produced by local fibroblasts. This process expedites clonal selection and expansion of CD8+ effector T cells (TEFF) by increasing the likelihood for TN to find and engage with cognate pMHC-presenting DCs. Here, we describe a novel function for CCR7 on CD8+ T cells in regulating TEFF generation. In addition to its role in enabling efficient T cell-DC encounters, CCR7 promotes T cell disengagement from DCs at late stages of cognate interactions. Mechanistically, CCR7 signals drive the promigratory Rac activator DOCK2 away from the late T cell-DC interface to induce T cell detachment.

CCR7-mediated T cell uncoupling from DCs correlates with rapid onset of proliferation and differentiation of TEFF characterized by high effector function and low PD1 expression. In turn, absence of a CCR7 “rheostat” causes protracted T cell-DC interactions and TCR signal integration, yielding dysfunctional TEFF that express markers associated with impaired T cell function and with reduced *in vivo* persistence. In sum, our results shed light on the physiological control of TCR signal duration and identify a key role for lymphoid tissue-expressed CCR7 ligands to prevent dysfunctional T cell activation, with potential ramnifications for the design of in vitro T cell activation protocols used in adoptive cell therapy.

P16

Characterization of the interaction between Diffuse large B cell lymphoma and NK cells by Two photon intravital microscopy

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Background

Non-Hodgkin lymphomas, especially diffuse large B cell lymphoma subtype (DLBCL), have posed significant medical challenges for several decades. Abnormal B cells grow in the lymphatic system, leading to tumor development in secondary lymphoid organs. Recently, genetically engineered immune cells like chimeric antigen receptor T and Natural killer (NK) cells, have emerged as potential therapies for the treatment of B-cell malignancies. However, the dynamics between immune cells, tumor and its microenvironment are not yet fully understood.

Methods

In this study, we used two-photon intravital microscopy to investigate the interaction between NK and tumor cells in a syngeneic mouse model for Eμ-myc induced B cell lymphoma.

Results

Our results showed that NK cell density increases in the inguinal lymph node (LN) upon lymphoma arrival. Applying image analysis techniques, we observed a significantly shorter length of movement, a shorter displacement, and a matching speed of NK cells in Eμ-myc lymphoma invaded LN versus control homeostatic LN. NK cells in the DLBCL microenvironment also exhibited the highest directionality and the lowest arrest coefficient. Additionally, we employed an action recognition algorithm to reveal a decrease in the percentage of NK cells exhibiting an arrested phenotype, while increases in flowing, patrolling, and directed NK cell phenotypes were identified.

Conclusions

In conclusion, our study demonstrates that two-photon intravital microscopy is a suitable technology for investigating immune-cancer cell interactions and provides insight into how hematological malignancies, specifically DLBCL, progress in the LN with significant alterations in NK cell dynamics. The described approach will aid in the assessment of therapies, and the identification of novel cancer-associated mechanisms *in vivo*.

P17

In vivo real-time imaging of afferent lymphatic vessels and leukocytes in tumors at the single-cell level

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Background

Afferent lymphatic vessels (LVs) serve as immunologic links between peripheral tissues and lymph nodes (LNs), facilitating antigen transport, immune cell migration from the periphery to draining lymph nodes (dLNs) and induction of adaptive immunity. Dendritic cells (DCs) and T cells are the main cell types migrating via this route. At the level of the lymphatic capillaries, DCs and T cells actively intravasate into the LV lumen via the discontinuous lymphatic endothelial cell (LEC) junctions and spend hours crawling, patrolling and interacting with capillary LECs and with each other. Upon reaching the downstream lymphatic collectors, DCs and T cells detach from the LV wall and are passively transported by the lymph flow to the dLNs. Apart from mediating cell and antigen transport, LECs have recently also been shown to express immunomodulatory molecules and to function as antigen-presenting cells. In the tumor context, LECs have emerged as important regulators of anti-tumor immunity. However, in contrast to studies in steady-state and inflamed murine skin, leukocyte migration and interaction with(in) afferent LVs in tumors have thus far not been studied at the single-cell level.

Methods

Using amelanotic B16.F10 melanoma tumor cells injected in Prox1-mOrangexCD11c-YFP and Prox1-mOrangexCD2-RFP reporter mice we imaged small tumors, tumor-associated LVs, CD11CYFP$^+$ DCs and CD2-RFP$^+$T cells in the murine ear skin using confocal and two-photon microscopy performed *in vivo* or *ex vivo* in whole-mounts of tumor-bearing mice. Using B16.F10-mCherry cells we were also able to visualize fluorescent tumor cells in the ear skin.

Results

In contrast to ear skin which was uninjected or distant from the tumor, several CD11c-YFP$^+$DCs and CD2-RFP$^+$-T cells were found to accumulate in the tumor and in proximity of tumor-associated LVs. A large number of leukocytes were further found interacting with each other and with the LECs lining the LVs, and found to have transmigrated into the LV and to be patrolling or arresting within the LV lumen.

Conclusions

Overall, by using intravital or whole-mount immunofluorescent microscopy, we were able to visualize and study leukocyte migration and their interaction with LECs in the tumor microenvironment with cellular resolution. This project sets the basis for future quantitative assessment of leukocyte interactions with tumor LVs and for studying the occurrence and significance of LEC-mediated immunomodulation.

P18

Role of plasmin-mediated cleavage of CCL21 by lymphatic endothelial cells in the regulation of the peri-lymphatic CCL21 gradient formation

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Background

Dendritic cell (DC) migration through afferent lymphatic vessels is highly dependent on the chemokine CCL21, which is produced by lymphatic endothelial cells (LECs), and on its DC-expressed receptor CCR7. While full-length CCL21 is known to form an immobilized peri-lymphatic chemokine gradient that reportedly attracts DCs into afferent lymphatics, also a soluble, C-terminally truncated CCL21 variant with potent chemotactic activity (CCL21-ΔC) has recently been described. How CCL21 is cleaved *in vivo* and how both protein variants act in concert to regulate lymphatic migration it is not fully understood.

Methods & Results

Here, we found that in inflammation the immobilized CCL21 gradient surrounding murine dermal lymphatics was diminished, whereas increased levels of soluble CCL21-ΔC were present in tissue extracts. At the same time, we observed that levels of plasminogen (plg) and of its cleavage product plasmin (plm), which was recently shown to cleave human CCL21 in vitro, were increased in inflamed as compared to resting skin. Moreover, we found that LECs *in vivo* and in vitro expressed several molecules important for the conversion of plg to plm, namely urokinase plg activator (uPA), its receptor (uPAR) and tissue plasminogen activator (tPA), suggesting a local connection between CCL21 expression and cleavage. Indeed, in an LEC-based in vitro assay, addition of plg and CCL21 to LECs resulted in CCL21 cleavage. Compared to full-length CCL21, the cleaved variant present in the culture supernatants was more potent in inducing DC chemotaxis in a 3D collagen migration assay, confirming it as CCL21-ΔC. CCL21 cleavage was reduced upon addition of inhibitors of plasmin or uPA or when performing the assay with LECs lacking uPA (uPA KO) or expressing a mutant uPA incapable of uPAR binding (uPA mut), demonstrating the involvement of plm/uPA/uPAR axis in this process. To study the effect of uPA-mediated CCL21 cleavage on DC migration, we compared the tissue localization of DCs in murine ear skin whole-mounts of WT, uPA KO and uPA mut mice by confocal microscopy. In line with the stronger chemotactic activity of CCL21-ΔC, significantly less DCs co-localized with dermal afferent lymphatics in uPA mut or uPA KO mice as compared to WT mice.

Conclusions

Overall, our data suggest that LECs, which at the same time are the producers of CCL21, are also involved in uPA-mediated conversion of plg to plm and the resulting cleavage of CCL21, thereby fine-tuning the peri-lymphatic CCL21 gradient.

P19

Pathogenic eosinophilia during invasive fungal infection via GPR35 sensing of platelet and mast cell 5-hydroxyindoleacetic acid

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Cryptococcus neoformans is the most common cause of fungal meningitis in humans yet understanding of immune mechanisms that determine the course of infection remains incomplete. During C. neoformans infection, pathogenic eosinophil recruitment occurs via type 2 inflammation, but we know little about the circuitry involved. Here we show that eosinophil recruitment to the lung during murine infection is promoted by the chemoattractant receptor GPR35 and its ligand, 5-hydroxyindoleacetic acid (5-HIAA). We define activated platelets and mast cells as key sources of GPR35 ligand activity. We demonstrate that loss of GPR35 from eosinophils, pharmacological inhibition of serotonin conversion to 5-HIAA, or genetic deficiency in 5-HIAA production by platelets and mast cells results in more efficient clearance of Cryptococcus. These findings provide evidence that 5-HIAA and GPR35 act as a novel eosinophil chemoattractant-receptor system that modulates the clearance of a lethal human fungal pathogen.

P20

Contextual Tissue Cytometry and its Applications in Immunology

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Background

While flow cytometry has been available for researchers and clinicians for decades to perform functional analyses on single cells and determine cellular phenotypes of large cell populations in blood, technologies to perform a similar analysis in situ – ie. in the tissue, the actual localization of most immune responses – are relatively new.

Methods

Our research teams at TissueGnostics and Queensland University of Technology have joined forces to combine TissueGnostics' existing tissue cytometry technology platform and established knowhow with innovative AI solutions to establish The Virtual Histopathologist. This represents a tissue cytometry platform that allows to quantify immune responses where they happen – in the tissue.

Results

Tissue Cytometry permits to determine the in-situ phenotype of individual cells as well as histological entities, like glands, vessels or tumor foci. Applications include but are not limited to the exploration of immune responses in situ and the tumor microenvironment and/or the spatial organization of cellular subpopulations. Earlier attempts to analyse single cells in tissue have mostly been subject to visual estimation, or – at best – to manual counting for decades. To better understand the function of inflammatory cells in tumor development, type and number of inflammatory cells and their proximity to glandular/tumor structures have to be analyzed in-situ and correlated with disease state. Using TissueFAXS™ Cytometry the time-consuming and error-prone human evaluation of stained histological sections can be approached with an observer-independent and reproducible technology platform, offering a high degree of automation, paired with user interaction at relevant points of the analytical workflow.

Conclusions

The TissueFAXS Cytometry platform incorporates Machine & Deep Learning algorithms. It can do end-point assays as well as live-cell imaging and time-kinetic experiments to measure enzyme activity. It also promotes tissue cytometry to a new level of quality, where complex cellular interactions, intracellular expression profiles and signal transduction cascades can be addressed on the single-cell level but still in histological context, empowering precision diagnostics.

P21

Leukemic Cell-Mediated Resistance to CAR-T Cell Immunotherapy

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Background

Treatment of relapsed B cell malignancies such as B cell Acute Lymphoblastic Leukemia (B-ALL) remains a significant clinical challenge. However, cell-based therapeutic approaches developed over recent years, most notably Chimeric Antigen Receptor (CAR) cytotoxic T (or CAR-T) cell immunotherapy, have made tremendous progress. Initial success of the use of CAR–T cells has been demonstrated targeting the surface antigen, CD19, on B cell cancers. Patients treated with CAR-T cell therapy for B-ALL can exhibit loss of disease burden acutely following treatment, with some patients achieving curative outcomes. However, many patients will relapse over time with tumors that are resistant to CD19 targeted CAR-T immunotherapy. A complete understanding of the mechanisms of CAR-T resistance is lacking; however, many relapsed B-ALLs demonstrate loss of surface expressed CD19. Receptor-mediated surface-loss of receptors and cognate ligands via intracellular endocytosis has been documented in both T and B lymphocytes. Further, the physical removal of surface antigens from target cells onto the surface of effector T cells, termed trogocytosis, has additionally been observed. The specific genes, pathways and cellular mechanisms that drive receptor-mediated surface loss remain elusive, particularly in the context of hematopoietic cancer cells.

Methods

To identify genes and pathways that play significant roles in surface loss of CD19 in B-ALL cancer cells, our laboratory has recently performed an *in vivo* whole genome CRISPR screen for mediators of resistance to CAR-T cell treatment. Following completion of the screen, many of the genes identified encode proteins involved in the cytoskeleton. Taking flow cytometry and confocal microscopy-based approaches, I have begun to assess the role of a select number of our target genes in CD19 loss from the B-ALL cell surface in response to CAR-T treatment.

Results

From our whole genome CRISPR screen we have identified several candidate genes that have significant roles in resistance to CAR-T cell immunotherapy. I have started to study and visualize surface loss of CD19 from the B-ALL surface as well as trogocytosis of CD19, in the context of a few candidate genes (e.g PAK2, FITM2)..

Conclusions

Results from these studies will further enhance our understanding of hematological cancer relapse to CAR-T treatment as well as develop further strategies for cellular immunotherapy approaches.

P22

Establishing a preclinical model of CAR-based phagocyte therapy for solid tumours

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Background

Engineering immune cells represents a promising strategy to treat cancer. For example, T cells modified to express a Chimeric Antigen Receptor (CAR) have revolutionized the treatment of blood cancer. A CAR is a synthetic receptor capable of specific antigen recognition and potent cell activation. Still, the limited abilities of T cells to enter and persist in the microenvironment of solid tumours (TME) has encouraged the development of new CAR-bearing cell therapies, such as those relying on mononuclear phagocytes (CARPHA). Indeed, among them, macrophages are one of the most abundant immune populations in solid tumours. These potent phagocytes have cytotoxic properties and can polarize the entire TME. Furthermore, macrophages bridge the innate and the adaptive immune systems, having the ability to present antigens, making them a good candidate for a new cellular therapy.

Previous work has shown CARPHA's ability to limit tumour progression in pre-clinical studies and their potential to remodel the TME. However, CARPHA's *in vivo* anti-tumour dynamics and precise mechanisms of action remain largely unknown.

Methods

Here, we have developed and started to validate preclinical models of CARPHA (colon carcinoma, melanoma, and subcutaneous lymphoma) with the aim to visualize CARPHA-immune cells cross talks during tumour regression. Bone-marrow mononuclear phagocytes are transduced to express a CAR recognizing a specific tumour antigen, and containing optimized intracellular transduction capacity for phagocytosis. This mechanism as well as apoptosis can be tracked *in vivo* and in real-time using a FRET-based probe expressed by the tumour cells.

Results

We have developed protocols to generate CAR phagocytes (CARPHA) from bone marrow derived macrophages or bone-marrow monocytes. *In vitro*, CAR-expressing macrophages were able to ingest different tumour types efficiently, albeit to different extent. CARPHA activity could be further monitored using a FRET-probe for phagocytosis expressed by tumour cells. In co-culture experiments, CARPHA efficiently reduced tumour load. Preliminary intravital imaging experiments showed that CARPHA can efficiently migrate to the tumour site, enter the tumoral mass and persist for up to 7 days post injection. In future experiments, we will evaluate the activity of CARPHA *in vivo*.

Conclusions

In sum, we have generated new preclinical models that are currently being used to explore the mode of action of this promising cell therapy.

P23

Fc-mediated phagocytosis triggers cytoskeletal rearrangement caused by RhoA oxidation

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Background

Despite phenotypic similarities, phagocytosis of pathogens and of apoptotic cells (efferocytosis) differ in their recognition mechanisms, engagement, and signaling pathways. While the initial stages of these processes are well studied, the cytoskeletal reorganization that follows particle uptake is not well understood.

Methods

We employed a variety of biochemical methods, in combination with confocal and fluorescence-lifetime imaging microscopy (FLIM) techniques to investigate the behavior of bone marrow-derived macrophages (BMDMs) and RAW264.7 cells, including triple Ezrin-Radixin-Moesin (ERM) knockout lines, as well as human neutrophils, to study the consequences of phagocytosis of distinct particles.

Results

Our results indicate that Fc-receptor-mediated phagocytosis differs from efferocytosis in its effect on cytoskeletal dynamics. Phagocytosis of IgG-coated targets that are recognized by Fc-receptors induced changes in motility, ruffling and macropinocytosis, and caused the disappearance of podosomes. These changes were associated with increased tension on the plasma membrane exerted by the cortical cytoskeleton. The increased tension was attributable to activation of RhoA.

We next investigated the role of proteins that anchor the cortical skeleton to the plasma membrane. Deleting ERM proteins attenuated the effects of Fc-receptor-mediated phagocytosis. ERM triple-knockout cells failed to round up and were able to retain podosomes after phagocytosis.

The activation of the RhoA was missing in macrophages ingesting phosphatidylserine-coated (efferocytic) targets. Unlike Fc-receptor mediated phagocytosis, efferocytosis fails to activate the respiratory burst. We concluded that the activation of RhoA is executed through the oxidation of its redox-sensitive motif by reactive oxygen species generated by the NADPH oxidase.

Conclusions

Our study highlights the intricate interplay between phagocytosis, cytoskeletal dynamics, and redox signaling, and their importance in the immune response. By oxidizing and activating RhoA, proinflammatory phagocytosis affects the cytoskeleton, which in turn conveys the increased tension to the plasma membrane via ERM proteins.

The resulting arrest in migration ensures that the phagocyte remains in the general area where IgG-coated targets, including pathogens, are found and need to be cleared.

P24

Identification of lymphatic smooth muscle cells specific markers

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Background

The lymphatic vascular system is composed of a network of lymphatic vessels (LVs) and is present in most vascularized tissues of the body. Leukocytes use lymphatic vessels to exit peripheral tissues through afferent lymphatics or lymph nodes through efferent lymphatics. Lymph nodes are important for immunosurveillance and the initiation of adaptive immunity, and their proper function highly depends on the ability of lymphatic vessels to efficiently propagate soluble antigens and leukocytes towards and out of the nodes. The contraction of lymphatic vessels, also known as lymphatic pumping, occurs in the collecting vessel segments and is initiated by lymphatic vessel smooth muscle cells (LV-SMCs), which surround the collecting vessels. Lymphatic pumping is regulated in the absence of external stimuli, similarly to the pacemaker cells of the heart, initiating contractions of the lymphatic vessels and maintaining this pumping activity. While it is known that LV-SMC differ from other SMC types in several aspects, no LV-SMC specific marker has thus far been identified, which would allow to better study this unique population.

Methods

To identify such markers, we FACS-sorted SMCs from lymphatic flank collectors and blood vessels of myosin heavy chain 11-GFP transgenic reporter mice and performed single cell RNA sequencing. Data analysis suggested that SMCs subdivide in three clusters, likely representing SMCs of arteries, veins and lymphatic collectors, and express a number of genes with distinct levels of gene expression. In order to identify LV-SMCs from the other types SMCs, we performed immunofluorescent stainings which confirmed that LV-SMCs form a specific cluster and express a number of markers that are absent on SMCs surrounding either arteries or veins.

Results

Preliminary data suggest that Slc22a1 (*Oct1*), which is an organic cation transporter, could be a potential specific marker for LV-SMCs.

Conclusions

These results provide the possibility to functionally investigate this previously uncharacterized LV-SMC population, using a reporter mouse model or specific inhibitors for Slc22a1.

P25

Swarming-on-a-chip imaging platform reveals metabolic requirements of neutrophil crowding

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Background

Neutrophil swarming is a highly coordinated population response of many individual neutrophils to infection and inflammation. Sequential phases of chemotaxis are followed by neutrophil cluster formation, which focalizes the microbicidal activity of a whole neutrophil population. By combining intravital microscopy and in vitro techniques, we could recently identify a cell-intrinsic mechanism that stops neutrophil swarming (Kienle et al., Science 2021).

Methods

The limited accessibility of mammalian tissues has so far complicated a detailed understanding of the cellular processes involved in neutrophil cluster formation. Utilizing a custom-build *in vitro* platform for high-quality live confocal fluorescence imaging of neutrophil crowding in response to bacterial stimuli, we are now able to tackle questions on single-cell and subcellular level and relate this information back to the swarming behavior of the whole neutrophil population.

Results

We use this platform to address fundamental biological questions, including the metabolic requirements of neutrophil crowding. Neutrophils are considered to be highly glycolytic cells and we can for example visualize the uptake of glucose during swarm initiation. The accessibility of this platform additionally allows us to progress our understanding of neutrophil metabolism during swarming by interfering with single metabolic pathways and enzymes. We visualize effector functions like ROS production and explore their dependency on specific metabolic pathways.

Conclusions

Using this swarming-on-chip imaging platform, we discovered an intricate balance between utilizing external glucose and internal energy storages, which is essential to meet the energy demands of neutrophils during homeostasis and swarming.

P26

Patient-derived slice culture systems – tool to understand the dynamic of immune cell lineages in cancers

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Background

Cancer evolves within a complex tissue environment consisting of a broad range of cells such as immune cells, stromal cells, and non-cellular components (e.g. cytokines)[1,2]. These networks known as the tumor microenvironment (TME) influence tumor progression, thus affecting therapeutic outcomes. Several lines of evidence strongly infer that the composition within these cancer-immune ecosystems is diverse and dynamic[3,4]. This highlights the urgent need to monitor distinct immune cell subsets on a real time basis. Which immune cells populate the tumor and how do they interact? Assessing these questions might help us to manipulate the immune system to fight cancer.

Methods

We utilized a murine tumor slices culture system to tag and track cytotoxic T-cells by fluorescently labelled α-CD8 antibodies. Subsequently, confocal live cell imaging was performed for 48h. IMARIS software was used for tracking analysis. In addition, we established a patient-derived slice culture system for head and neck cancer (HNSCC). Cellular composition of this HNSCC ex vivo cultures was characterized by multiplex staining. T-cell function was confirmed by flow cytometry. Additionally, we tracked cytotoxic T-cells in patient-derived HNSCC slice cultures for 24h.

Results

Here, we established a stable 4D live cell imaging of cytoxic T-cells within murine tumor slice cultures. Subsequent image analysis revealed three different motility patterns, pointing to a different motility status of the T-cells (e.g. exhausted or highly motile). Additionally, we established a patient-derived slice culturing system for HNSCC, preserving the individual nature of a patient cancer-immune ecosystem following 48hrs of cultivation[5]. Tumor-intrinsic T-cells were still viable and could be activated by α-CD3/α-CD28 stimulatory antibodies. Next, we performed live cell tracking of CD8 +ve T-cells for 24h. These results point to similar motility pattern as observed in the murine system, although the majority of the cytoxic T-cells showed a motionless state. Future experiments will uncover whether these static T-cells could represent an exhausted phenotype, thereby not capable to attack the tumor.

Conclusions

In conclusion, the established 4D live cell imaging approach within patient-derived HNSCC slice cultures could constitute a versatile tool to not only study the dynamic interplay of distinct immune cell populations, but also to uncover the mechanisms of action of cancer-immunomodulatory substances.

P27

Phenotypic diversity of nasal mucosa neutrophils is linked to specialization of function

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Background

The nasal cavity traps airborne pathogens in mucus to prevent them to reach the lungs. Once immobilized, these pathogens are subsequently targeted by immune cells that reside in the nasal mucosa and that are key to preventing local infection. Remarkably, the composition of immune cell populations that reside in the nasal mucosa, their origin, and their anti-microbial effector functions are questions that have remained largely unexplored.

Methods

We employed initially flow cytometry and IHC of nasal tissue to identify and quantify different immune cell populations in the nasal mucosa. Subsequently, we developed a 2-photon intravital microscopy approach to visualize these cells and focused specifically on neutrophils as we noticed they were a prominent cell population in this tissue. Microsurgery allowed us to gain direct access to the nasal mucosa of living mice to trace extravascular neutrophils in the steady state and during infection. These extravascular neutrophils were quantified and characterized into different subpopulations by flow cytometry and RNAseq analysis. Finally, we used parabiosis to establish the different populations' origins and we tested their antimicrobial abilities.

Results

Large numbers of neutrophils were observed in the extravascular milieu of the nasal mucosa at steady state. These extravascular neutrophils were phenotypically subdivided into three distinct populations, termed N1, N2, and N3, based on cell surface markers and distinct mRNA expression profiles. Through parabiosis and imaging, we determined that N1 neutrophils resided in the bone marrow adjacent to the nasal cavity, possibly migrating via bone conduits directly into the adjacent nasal mucosa. By contrast, N2 neutrophils were determined to enter the nasal mucosa from the blood. Newly homed N2 cells persisted in nasal tissue for at least 6 days and, during this period, differentiated into N3 neutrophils, with the former expressing genes commonly ascribed to conventional neutrophils, whereas the latter upregulated genes commonly ascribed to antigen-presenting cells. Remarkably, while N2 neutrophils were the principal subset that phagocytosed bacteria upon local infection, N3 neutrophils engulfed material from surrounding cells in the steady state, in addition to presenting antigens to CD8+ T cells in vitro.

Conclusions

The nasal mucosa encompasses a constitutive population of extravascular neutrophils that display diverse origins and specialization of function.

P28

Real time immersion into the mode of action of anti-CD20 antibody in different anatomical sites: same therapy different outcomes

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Background

The use of monoclonal antibodies (Ab) to eliminate malignant cells can be an effective strategy to treat patients with cancer. Rituximab, an anti-CD20Ab, was the first therapeutic Ab to be used and has significantly improved the outcome of patients with B cell malignancies. It acts by depleting normal and malignant B cells through several possible mode of action (MOA) extensively explored in vitro: Anti-CD20Ab trigger Ab-dependent cellular cytotoxicity (ADC) by natural killer (NK) cells, bind the complement cascade (CDC) or induce Ab-dependent phagocytosis (ADP) by macrophages. *In vivo*, target cells can invade multiple sites [eg. blood, bone marrow (BM), lymph nodes (LN)] and thus different MOA may occur depending on the effector composition of the anatomical site. There is a general assumption that anti-CD20mAb anti-tumor activity largely relies on FcR-dependent MOA further supported by patients' data, associating polymorphisms in Fc receptors with improved therapeutic response. Despite NK cells being often considered as the central players, depletion of macrophages/monocytes in mouse models has highlighted them as essential for mediating Ab therapeutic activity. Nevertheless, despite two decades of clinical use, linking the respective contribution of these MOA in different sites, to the therapeutic response of anti-CD20Ab, remains a central question to optimize Ab use in the clinic.

Methods

Through 2 photon real time *in vivo* imaging, we investigate anti-CD20Ab in three common sites of B cell malignancies by using fluorescent mouse-models and lymphoma cells expressing a dual FRET-based reporter of apoptosis and phagocytosis.

Results

We visualized target B cells travelling through the liver sinusoids and being depleted efficiently by Kupffer cells within minutes. By contrast, tumor cells in the BM or LN appeared largely sessile highlighting the importance of evaluating local MOAs. Like in the liver, we find ADP by macrophages to be the dominant MOA but with a reduced efficacy: B cell tumor elimination in the BM was partial with ADP being no longer active after one hour. Moreover, macrophages were present at low density in tumor-rich regions and could only reach out for neighboring tumors further impeding anti-CD20Ab activity.

Conclusions

Here, we pinpoint both temporal and spatial constraints limiting ADP in the BM, raising important questions for treatment optimization. A fine understanding of MOA at different tumor sites is key for the rational design of next-generation therapies.

P29

Cellular dynamics of tingible body macrophages locally activated by apoptotic cells in the germinal centre

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Background

Germinal centres (GCs) that form within lymphoid follicles during antibody responses are sites of massive cell death. Tingible body macrophages (TBMs) are tasked with the clearance of these apoptotic cells to prevent secondary necrosis and autoimmune activation by intracellular self-antigen. Indeed, defects in the clearance of apoptotic cells by TBMs results in autoantibody formation against nuclear self-antigens and lupus-like disease. Despite their critical housekeeping function, little is known about TBMs based on static analysis of ex vivo tissue sections that provide only a rough two-dimensional snapshot in time.

Methods

We developed an *in vivo* TBM mouse model and used intravital two-photon microscopy, photo-conversion lineage tracing and two-photon photoablation, to track the origin and cellular dynamics of apoptotic cell clearance by TBMs.

Results

Within immunised lymph nodes, the CD169Cre reporter marked TBMs in GCs that had engulfed apoptotic B cell fragments (‘tingible bodies’), in addition to the resident subcapsular sinus (SCS) and medullary sinus macrophages. Intravital two-photon microscopy revealed that these TBMs were non-migratory, but used highly dynamic cellular processes to chase and capture motile dead cell fragments. We also observed marked follicular macrophages in resting lymph node follicles that contained few ‘tingible bodies’. Inducing apoptosis of B cells in the follicle using two- photon photoablation locally activated these follicular macrophages to engulf the apoptotic cells, causing them to acquire a TBM-like morphology. Lineage tracing using whole lymph node photo-conversion showed that TBMs derive from a tissue-resident macrophage population and blocking colony stimulating factor-1 receptor (CSF1R) depleted SCS and medullary sinus macrophages, but not resting follicular macrophages and TBMs. Single cell transcriptomics identified a TBM population that upregulated genes involved in apoptotic cell clearance following immunisation.

Conclusions

Apoptotic B cells in early GCs likely trigger the activation of follicular macrophages into classical TBMs to clear apoptotic debris and prevent antibody-mediated autoimmune diseases.

P30

Response to primary chemoradiotherapy of locally advanced oropharyngeal carcinoma is determined by the degree of cytotoxic T cell infiltration within tumor cell aggregates

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Background

Effective anti-tumor immune responses are mediated by T cells and require organized, spatially coordinated interactions within the tumor microenvironment (TME). Understanding coordinated T-cell-behavior and deciphering mechanisms of radiotherapy resistance mediated by tumor stem cells will advance risk stratification of oropharyngeal cancer (OPSCC) patients treated with primary chemoradiotherapy (RCTx).

Methods

To determine the role of CD8 T cells (CTL) and tumor stem cells for response to RCTx, we employed multiplex immunofluorescence stains of the antigens pan-Cytokeratin, Ki67, CD271, CD8, PD-L1 and p16INK4a on pre-treatment biopsy specimens from 86 advanced OPSCC patients and correlated these quantitative data with clinical parameters. Multiplex stains were analyzed at the single-cell level using QuPath and spatial coordination of immune cells within the TME was explored using the R-package Spatstat.

Results

Our observations demonstrate that a strong CTL-infiltration specifically into the epithelial tumor compartment (HR for overall survival, OS: 0.35; $p<0.001$) and the expression of PD-L1 on CTL (HR: 0.36; $p<0.001$) were both associated with a significantly better response and survival upon RCTx. By contrast, overall CTL infiltration, regardless of the affected compartment, was not associated with response or survival upon RCTx. As expected, p16 expression was a strong predictor of improved OS (HR: 0.38; $p=0.002$) and correlated with overall CTL infiltration (r: 0.358, $p<0.001$), highlighting the strong immunogenicity of HPV-infected tumors. Finally, OPSCC proliferative activity and the expression of the tumor stem cell marker CD271 within the tumor cell compartment were not found to be prognostic.

Conclusions

In this study, we could demonstrate the clinical relevance of the spatial organization and the phenotype of CD8 T cells within the TME. In particular, we found that the infiltration of CD8 T cells specifically into the tumor cell compartment was an independent predictive marker for response to chemoradiotherapy, which was strongly associated with p16 expression. Meanwhile, tumor cell proliferation and the expression of stem cell markers showed no independent predictive effect in response to RCTx and require further study. These findings have important implications for future stratification of patients for more or less aggressive treatment approaches.

P31

Cellular architecture shapes the naïve T cell response

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Background

Naïve T cells display robust population-level responses following antigen stimulation, characterized by proliferation and differentiation into diverse subsets. This robustness arises from individual T cells pursuing various differentiation trajectories following T cell receptor (TCR) signaling. However, the cell-intrinsic determinants controlling these single-cell decisions remain enigmatic.

Cell morphology and the intracellular organization of organelles, here collectively referred to as cellular architecture, influence cell signaling and function across a variety of systems, including immune cells. Thus, we sought to investigate the potential link between T cell architecture and single-cell response decisions.

Methods

We developed a platform that simultaneously measures single-cell architecture, function and phenotype, using automated fluorescence microscopy and deep learning-based image analysis combined with live T cell functional assays using both human and murine models.

Results

Our findings reveal that T cell architectural heterogeneity is defined by the presence ($T_{Ø}$) or absence (T_{O}) of nuclear envelope invaginations established during thymic maturation, and dynamically coordinated throughout activation and differentiation, with establishment of CD8 T cell effector phenotypes being characterized by loss of $T_{Ø}$ and gain in morphological polarization. Upon TCR stimulation of monoclonal CD8 naïve T cells, $T_{Ø}$ architectures display increased TCR signaling, heightened and more sustained store-operated calcium entry, and faster and stronger expression of early response genes than T_{O}. This increased response efficiency is explained by higher baseline expression of STIM1 and the spatial concentration of key cellular machinery.

Conclusions

Our data redefine the textbook on primary T cell morphology, identifying subcellular architecture as a cell-intrinsic and actively regulated mechanism by which individual naïve T cells predetermine their responsiveness, with significant consequence in the fields of T cell differentiation, immunotherapy, and aging.

P32

Single protein mapping of immune checkpoints reveals CD80 as an antagonist of MHC-I/PD-L1 clusters

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The activation and function of immune cells is guided by signals encount ered on the surface of their interaction partners. In the case of T cells, activatory (costimulatory) and inhibitory (checkpoint) molecules engaged on the surface of dendritic cells during initial T cell activation dictate whether T cells become effectors, while the relative ratios of these molecules determine if effector T cells can subsequently kill their targets, such as tumor cells. Despite the importance of these molecules in T cell immunoregulation, little is known about the spatial organization and surface architecture of these proteins.

To address this knowledge gap, we employed multiplexed, super-resolution DNA-PAINT imaging on the surface of individual mouse cDC1s and melanoma cells to simultaneously map the single protein distributions of 6 different proteins: the co-stimulatory molecules CD80 and CD86, the immune checkpoints PD-L1 and PD-L2, and MHC-I and MHC-II.

Across the course of cDC1 activation, we validated previously reported, in addition to identifying multiple unreported, protein-protein interactions, with the DC surface enriched for smaller protein nano-clusters.

In striking contrast to cDC1s, melanoma spatial organization was dominated by large-scale PD-L1 and MHC-I aggregates. Critically we identify CD80 as a key factor dictating whether cells adopt a “melanoma-like” or “DC-like” surface architecture. CD80 ablation from cDC1s precipitated melanoma-like PD-L1/MHC-I surface aggregates, while CD80 over-expression on melanoma cells led to a dispersed, DC-like surface organization.

Collectively, these data reveal unappreciated and complex spatial organization associated with immune activation vs repression, and identify CD80 as a key “remodeling” factor that disrupts formation of immunosuppressive surface complexes. Our study is thus an important stepping stone towards the much higher plex, bona fide “spatial proteomics” technologies required to comprehensively address the many unanswered fundamental biology questions surrounding cell surface organization.

P33

CD103 regulates dermal Regulatory T cell motility and interactions with CD11c-expressing leukocytes to control skin inflammation

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Background

Dermal regulatory T cells (Tregs) are essential for the maintenance of skin homeostasis and control of skin inflammatory responses. In mice, Tregs in the skin are characterized by high expression of CD103, the αE integrin. Evidence indicates that CD103 promotes Treg retention within the skin, although the mechanism underlying this effect is unknown. The main ligand of CD103, E-cadherin, is predominantly expressed by cells in the epidermis. However, as the majority of Tregs are located within the dermis, the nature of the interactions between E-cadherin and CD103-expressing Tregs is unclear. The aim of this study was to use multiphoton intravital microscopy (MP-IVM) to examine the contribution of CD103 to Treg behaviour in resting and inflamed skin

Methods

Skin inflammation was induced using oxazolone-induced contact hypersensitivity (CHS). Tregs were imaged in Foxp3-GFP Treg reporter mice, using MP-IVM of flank skin, in some cases in combination with CD11c-YFP reporter mice to detect antigen-presenting cells.

Results

Inhibition of CD103 in uninflamed skin did not alter Treg behaviour, while in contrast, 48 h after inducing CHS by oxazolone challenge, CD103 inhibition increased Treg migration. This coincided with E-cadherin upregulation on infiltrating myeloid leukocytes in the dermis. Using CD11c-EYFP x Foxp3-GFP dual reporter mice, inhibition of CD103 was found to reduce Treg interactions with dermal dendritic cells, affecting both the duration of interactions and the percentage of Tregs undergoing interactions. CD103 inhibition also resulted in increased recruitment of effector T cells and monocyte-derived dendritic cells in challenged skin.

Conclusions

These results demonstrate that CD103 controls intradermal Treg migration, but only at later stages in the inflammatory response, when E-cadherin expression in the dermis is increased, and provide evidence that CD103-mediated interactions between Tregs and dermal dendritic cells support regulation of skin inflammation.

P34

Profiling the impact of estrogen receptor beta on the inflammatory colonic immune microenvironment using the COMETT™ platform for multiplex immunofluorescence

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Background

Chronic inflammation promotes the development of colorectal cancer (CRC). Studies show a protective effect of estrogen, mediated by estrogen receptor beta (ERβ), against the development of CRC. We have shown that intestinal ERβ (iERβ) protects against colitis and CRC by modulating inflammatory signaling and that iERβ modulates the tumor immune microenvironment. Deletion of iERβ impaired natural killer cell infiltration and increased macrophage and T cell infiltration into tumors. The aim of this study was to investigate how iERβ affects the immune microenvironment during colitis.

Methods

Wild-type (WT) mice and mice that lack iERβ (ERβKOVil mice) were treated with vehicle or AOM/DSS for 9 weeks to induce colonic inflammation and early carcinogenesis. Using the novel COMET™ platform (Lunaphore Technologies) for multiplex immunofluorescence, we stained formalin-fixed, paraffin-embedded Swiss-rolled colons from 32 mice for a panel of 10 markers. QuPath was used to analyze the images and quantify the results. We profiled the numbers of infiltrating natural killer cells (Klrb1c+), M1 (F4/80+CD86+) and M2 (F4/80+CD206+) macrophages, regulatory (FOXP3+), helper (CD3+CD4+), and cytotoxic (CD3+CD8+) T cells, neutrophils (Ly-6G+), and dendritic cells (CD11c+) in the colonic mucosa and muscularis externa (ME). The results were compared according to treatment, genotype, and sex.

Results

Our findings show that deletion of iERβ alters the colonic immune microenvironment during colitis and early carcinogenesis. Treated ERβKO^{Vil} mice had significantly higher numbers of all immune cell populations profiled infiltrating the ME, as well as significantly increased numbers of infiltrating pro-inflammatory M1 macrophages in both the mucosa and ME. Regulatory T cell infiltration into the ME was significantly higher in ERβKO^{Vil} mice compared to WT mice. ERβKO^{Vil} mice, especially males, also had significantly higher numbers of helper and cytotoxic T cells infiltrating the ME during colitis. Interestingly, treated WT males displayed no increase at all in the numbers of infiltrating M2 macrophages.

Conclusions

iERβ modulates the inflammatory immune microenvironment during colitis. Deletion of iERβ promotes the infiltration of pro-inflammatory macrophages and increases the numbers of immune cells infiltrating the muscular layer, likely exacerbating colitis. Activation of iERβ could be of potential use to treat colitis and prevent CRC.

P35

Non-invasive tracking of T cell recruitment to the tumor micro-environment in a murine glioma model by high field cellular MRI

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Background

Gliomas are the most common primary tumors of the central nervous system (CNS) with high malignancy and poor median survival. The development of new immunotherapies makes non-invasive imaging of immune cell dynamics of upmost importance in order to monitor and adapt immunotherapies accordingly, especially for malignancies that do not allow regular biopsies like brain tumors.

Methods

Iron oxide nanoparticle (NP) labeling of T cells was thoroughly characterized by flow cytometry, confocal microscopy and transmission electron microscopy (TEM). Intratumoral T cell distribution was visualized non-invasively by T2* relaxometry magnetic resonance imaging (MRI) at 9.4T. MRI-based intratumoral visualization of T cells was correlated with light sheet microscopy after whole-brain clearing, which enabled the generation of a 3D model of intracranial T cell distribution.

Results

We show that non-invasive tracking of adoptively transferred, tumorepitope specific T cells is possible by MRI in a murine glioma model using iron oxide NP without affecting T cell viability or functionality. T2* relaxation times of the tumor at an early time point after adoptive cell transfer (ACT) were indicative for tumor response or resistance, which demonstrates that non-invasive quantification of spatial and temporal T cell dynamics in the tumor microenvironment can function as a predictor for immunotherapy efficacy. Iron oxide NP labeling could furthermore be extended to murine as well as human CAR T cells and could therefore be translated to clinical adoptive cell therapies.

Conclusions

Intratumoral visualization of T cell distribution by MRI in a glioma model is possible and can serve as a predictor for response and resistance. Non-invasive tracking of immune cells could hence aid the preclinical development of new immunotherapies and function as a valuable prognostic marker for adoptive cell therapies.

P36

Obesity induced Type I IFN sensing by Kupffer Cells hampers liver regeneration after partial hepatectomy

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Background

Obesity affects billions of individuals worldwide and is a risk factor for poor liver regeneration after resection. The underlying mechanisms remain elusive. Hepatic macrophages were identified as key players for liver regeneration by the production of cytokines and growth factors (GF), and phagocytosis of apoptotic cells. Our work focusses on the impacts of obesity in macrophage function during liver regeneration after partial hepatectomy (PHx).

Methods

To address the effects of obesity in macrophages, we used a high fat diet (HFD)-induced obesity murine model. To this, after 12 weeks of feeding with HFD, mice were subjected to 2/3 PHx. To study the composition and replenishment of hepatic macrophage populations, we used fate-mapping models and bone marrow chimeras. To address the relevance of the different subsets of hepatic macrophages for liver regeneration, we used the Clec4F-DTR and MaFIA mice to deplete Kupffer cells (KC) or the monocyte-derived macrophages (moM) respectively. We conducted fluorescent microscopy analysis and mathematical modeling for clonai expansion on CX3CR 1 CreERT2/R26R-*Confetti* mice to address the proliferative capacity of KC in obese mice after PHx. Finally, we used bulk RNAseq to further understand the reprogramming KC undergo in obesity and during liver regeneration.

Results

We identified that KC, but not moM, are required far liver regeneration after PHx. We found a dramatic loss of KC in obese mice, which were replenished by moM. KC from obese mice show impaired cytokine and GF production, as well as reduced phagocytosis of apoptotic cells. KC from obese mice show impaired clonai expansion and proliferation, that correlates with reduced liver regeneration. RNA-seq indicateci an increase in IFNA receptor (IFNAR)-stimulated genes (ISGs) in KC from obese mice. BM chimera experiments with $IFNAR^{-/-}$ as donor were capable of rescuing KC numbers in obese mice. Finally, treatment of obese mice with anti-IFNAR blocking antibodies prior to PHx rescued liver regeneration in obese mice.

Conclusions

Our work has identified KC has a key player during liver regeneration after PHx. Obesity led to impairment of KC functions necessary far liver regeneration. In addition, obesity led to increase in Type I IFN signaling in the liver and increase ISGs expression in KC. With IFNAR blockade therapy we were able to rescue liver regeneration in obese mice, highlighting a potential new target for therapeutic intervention in patients.

P37

The immune response in wound healing and cancer

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Background

The skin's immune system plays a critical role in maintaining this barrier function and responding to any injuries or infections that may occur. When the skin is injured a complex series of events is triggered to promote wound healing. This process involves inflammation, tissue regeneration, and tissue remodelling, with different cells and molecular mediators playing specific roles in each stage of the process. Understanding the underlying mechanisms of wound healing is crucial for developing effective treatments for a range of conditions, from minor cuts and bruises to chronic wounds and even cancer.

Methods

In this study, we used state-of-the-art techniques to investigate the role of immune cells, specifically neutrophils, in both regenerative wound healing and non-healing wounds such as tumours. We employed intravital microscopy, which allows for real-time visualization of cellular processes in living tissue, and single-cell RNA sequencing, which enables the identification and characterization of individual cells and their gene expression profiles.

Results

We took advantage of our expertise in imaging neutrophils *in vivo* to provide, for the first time, a detailed understanding of how neutrophil functions evolve over time during wound healing (from tissue-injuring to tissue-restoring) and tumor progression (from anti-tumor to pro-tumor). Our data suggests that neutrophils survive in the skin for extended period of times after injury, and have a distinct phenotype, migration, and function. Moreover, our study demonstrated that changing the TME from a chronic, aberrant wound healing response to acute microbe-triggered inflammation induced extensive changes in tumor neutrophil transcription, migration, and function and ultimately repressed tumor growth in a neutrophil-dependent manner.

Conclusions

Our research has shed new light on the complex interplay between neutrophils and the immune system in the context of both wound healing and cancer. By understanding how these cells function and evolve over time, we can develop new therapies and interventions to control their activities and promote healthy tissue repair. Our findings may have significant implications for the development of novel treatments for chronic wounds and cancer, two conditions that share many similarities in terms of their underlying biological mechanisms.

P38

Visualizing and quantifying resident memory CD8 T cell immunosurveillance and CD4 T cell target elimination within the mouse female reproductive tract

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Background

Resident memory T cells (TRM) constitute a recently identified lineage that remains permanently positioned in non-lymphoid tissues. TRM have emerged as principal surveyors within tissues and in coordination with other immune cells accelerate pathogen clearance. Identifying the minimum number of CD8 TRM required to comprehensively survey their tissue of residence within a finite period would enable the detection and eradication of rare infected or malignant cells and serve as an important step in the rational design of T cell-based vaccines. Here, we determined the density of CD8 TRM required for comprehensive immunosurveillance of the murine female reproductive tract and compared it to the density of genital HIV-specific CD8 memory T cells elicited by a T cell inducing vaccine in the STEP trial which failed to protect vaccinees from HIV acquisition.

Methods

To quantify immunosurveillance requirements at steady state, we performed intravital imaging and visualized CD8-CD4 TRM interaction in the murine uterus. We also tested the *in vivo* cytotoxic capacity of CD8 TRM against CD4 T cells presenting cognate antigen that were injected into the murine uterus to model the killing of HIV-infected cells.

Results

Based on CD8-CD4 contact rates obtained from 15 animals, surveillance efficiency was directly correlated with the density of antigen-specific CD8 TRM but not with target cell density. In the murine uterus, 353 antigen-specific CD8 T cells/mm^3 or ~2 antigen-specific CD8 T cells/mm^2 in 6μm thin sections would be required for complete tissue immunosurveillance within 2 days. In contrast, vaccinees from the STEP trial had ELISPOT readings of ≥55 spot-forming units/10^6 blood leukocytes or an estimated 0.04 genital HIV-specific CD8 T cells/mm^2 in 6μm sections. Our mouse-derived CD8 TRM surveillance data predicts 50-fold suboptimal density of HIV-specific CD8 T cell in the STEP trial. Upon recognition of CD4 T cells bearing cognate antigen, CD4-CD8 TRM formed long LFA-1/ICAM-1-dependant kinapses that enabled CD8 TRM pursuit of motile CD4 targets, and reduction in target cell velocity. CD8 TRM initiated the elimination of target cells by 3 hours.

Conclusions

We conclude that the magnitude of CD8 TRM is a crucial determinant of tissue immunosurveillance. Vaccines that generate abundant CD8 TRM in non-lymphoid tissues may have improved efficacy through rapid immunosurveillance and target cell elimination.

P39

Dynamic Tregs suppress CD8 T cells while migrating in the vicinity of a CXCR3-dependent niche

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The fine-tuning of the antiviral immune response by Tregs is crucial for protecting the host tissue from collateral damage while ensuring successful pathogen clearance. Here, we set out to elucidate the spatiotemporal dynamics of Treg-mediated control of CD8 T cell differentiation and expansion during acute viral infection. Using scRNAseq, analysis of tissue sections, 2-photon intravital microscopy and various genetic mouse models, we identified local niches of Treg-mediated control of CD8 T cells in lymph nodes. Following an initial activation of Tregs by CD4 T cell-derived IL-2, Tregs regulate IL-2 availability, which becomes limiting between d3-d4 post infection. During this timeframe CXCR3-guided access of activated CD8 T cells to antigen-presenting cDC1 is essential to maximize CD8 T cell proliferation and effector differentiation. Tregs controlled this access dynamically, without arresting, while migrating in the vicinity of the cDC1 niche in a CXCR3-dependent manner. Conceptually, Tregs controlled the access to the IL-2-rich niche around cDC1 by limiting CXCL10 production and by consuming IL-2 thereby limiting its availability. Notably, while Tregs primarily counteract the function of CD4 helper T cells they are also able to control CD8 T cells in the absence of CD4 help. Therefore, we asked whether Tregs also control the proliferation of CAR T cells directed against endogenous B cells, which also don't receive helper signals. Indeed, Treg cells did not control CAR T cell expansion in line with the failure of CAR T cells to interact with cDC1. However, redirecting CAR T cells towards the cDC1 niche significantly enhanced their proliferation and depleting Tregs further improved B cell killing. Together, we defined spatiotemporal niche of Teff generation; how it is accessed and how it is controlled by Tregs.

P40

Arp2/3 preserves the survival and longevity of tissue resident mast cells

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Background

Mast cells (MCs) are long-lived tissue-resident immune cells with important roles during allergies and anaphylaxis. We showed that MCs use a slow and strictly substrate-dependent mode of interstitial tissue movement, which makes them unique among immune cells (Kaltenbach*, Martzloff*, Bambach* et al., Nature Immunol, accepted).
Actin dynamics and actin-related proteins (ARPs) control many aspects of immune cell movement. ARP2/3, a key actin nucleator complex, has been shown to modulate migration modes of several immune cell types. However, little is known about the role of ARP2/3 for the tissue residency of immune cells. Therefore, we studied the role of ARP2/3 for the homeostasis of tissue-resident MCs.

Methods

We used MC-specific knockouts of ARPC4 for the depletion of ARP2/3. Mouse strains carried fluorescent reporters to image MC shapes *in vivo* or MC actin dynamics in vitro. To study endogenous MCs in tissues, fixed ear skins of differently aged mice were imaged with confocal microscopy. Electron microscopy was used to characterize cell morphologies of in vitro cultured MCs. To mimic MC tissue dynamics, time-lapse video-based microscopy was established to observe MC dynamics in 3D in vitro matrices over 4-7 days. With fluorescent cell cycle probes, the longterm imaging of 3D MC dynamics provided a powerful method to dissect the mechanisms underlying the *in vivo* phenotype.

Results

Surprisingly, loss of ARPC4 leads to a gradual depletion of endogenous MC populations *in vivo*. While Arpc4$^{-/-}$ MCs switch from lamellopodia- to lobopodia-driven movement in 3D gels and move with increased speeds, this migration phenotype did not explain our *in vivo* findings. Instead, we found a crucial role for ARP2/3 in controlling MC proliferation in physiological tissues. Analysis of MC dynamics in adhesive 3D matrices revealed that Arpc4$^{-/-}$ MCs undergo a G1/S cell cycle block followed by massive cell death. Arp2/3 inhibition in primary human MCs in 3D matrices resulted in the same phenotype. Strikingly, the proliferation and survival of *Arpc4*$^{-/-}$ MCs can be rescued in vitro and *in vivo*, when the integrin-mediated interaction of MCs with ECM is interfered with.

Conclusions

Our findings highlight a key role of ARP2/3 in protecting the integrity of slow-moving MCs, which naturally adhere to their native tissue environment. Thus, we identify an unexpected physiological role of Arp2/3 for controlling the cell cycle and the longevity of tissue-resident MCs.

P41

Comprehensive 3D imaging and topological analysis of blood and lymphatic channel systems in entire murine lymph nodes

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Background

Lymph nodes (LNs) are a cornerstone of immunity, by providing critical environments for immune surveillance and the establishment of protective immune responses. Key to their function is a highly organized microanatomy, characterized by intertwined networks of blood and lymphatic channels transporting cells and antigen from the periphery, and specialized homing zones for the adequate stimulation of T and B cells. Intriguingly, a fine conduit network channels lymphatic fluid to compartments deep inside the LN and provides a scaffold for cell migration. Resolving these delicate structures and the dynamic and often organ-wide events leading to immune activation is directly linked to current imaging capabilities, yet achieving high-resolution imaging on an organ-wide scale remains a challenge.

Methods

We have piloted novel imaging technology to capture entire murine LNs using custom-engineered confocal-based systems with point- and line-scanning that achieve an isotropic voxel resolution of up to 0.5 µm. LN blood and lymphatic channels were labelled with fluorescent tracers and captured in their entirety, followed by custom image processing and analysis to obtain detailed network statistics. The quality of the acquired data allowed us to create a topology model of the conduit network within the T cell zone and use it as a ‘road map’ for the realistic agent-based modeling of T cell migration.

Results

Using novel 3D imaging technology we were able to capture the blood and lymphatic channel system in murine LNs at unprecedented detail and generated the first map of the conduit network across the entire T cell zone. Topological analysis revealed homogenous branching of the conduit channels but disclosed differences in density between the superficial and deep T cell zones, yet without affecting overall T cell motility. The extensive 3D imagery delivered a broad overview of the regions accessible to lymph-derived small antigen molecules, including the T cell zone conduits, perivascular sleeves, and intriguing clusters within B cell follicles we termed “follicular reservoirs”.

Conclusions

New advances in 3D imaging allowed us to create a unique vista of the labyrinthine channel systems within LNs at unprecedented scale and resolution. This technology now offers an opportunity for the dynamic tracking of antigen material across the entire organ, and the theoretical modeling of LN biology, to further advance our understanding of LN structure and function.

P42

Development of drug delivery system by using innate immune cells and nano-particles for treatment of Glioblastoma

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Background

Glioblastoma (GBM) is the most common cancer that originates in the brain and spinal cord. Glioblastoma is well known as the deadliest cancer, due to short survival life expectancy and low survival rate after diagnosis. There are several challenges in the treatment of glioblastoma, such as the difficulty of completely removing tumor tissue from the brain through surgery without provoking any matters in brain functions or the difficulty in drug delivery to glioblastoma due to the Brain-blood barrier (BBB). To overcome these challenges, we suggested neutrophils as a drug-delivery vehicle to target and treat glioblastoma. Neutrophil is an innate immune cell that migrates to the inflammatory region and could function as a natural vehicle of drug that can target glioblastoma.

Methods

To make a glioblastoma xenograft mouse model, we established implantation of glioblastoma by injection of mouse glioblastoma cell, GL261, into the mouse brain using stereotaxic. Subsequently, we isolated GFP+ neutrophils from LysM-GFP mice and adaptively transferred them to glioblastoma-formed mice and also healthy mice by i.v. injection. By using flow cytometry, we confirmed the population of adaptively transferred neutrophils, which migrate to the mouse brain. Furthermore, using two-photon intravital microscopy, we identified whether adaptively transferred neutrophils migrate to the mouse brain in real time.

Results

Consequently, we observed a larger number of neutrophils migrated to glioblastoma area of the mouse brain rather than the healthy mouse brain by flow cytometry. In addition, using two-photon intravital imaging, we confirmed that adaptively transferred neutrophils could penetrate BBB and migrate to the mouse brain, especially to glioblastoma, using two-photon intravital imaging. We suppose that adaptively transferred neutrophils could penetrate BBB and provided as a better clinal treatment for glioblastoma.

Conclusions

Collectively, our study suggests a strategy of treatment for glioblastoma which may overcome the hurdle that the drug hardly penetrates BBB, enabling better treatment of glioblastoma. Furthermore, we expect neutrophils could recruit other immune cells such as effector T cells, and enhance the effectiveness of glioblastoma treatment.
Henceforward, we will provide further studies of the effectiveness of clinical treatment by adaptively transferring drug-delivered neutrophils to glioblastoma xenograft mouse model.

P43

WNK1 controls immune synapse formation in cytotoxic T cells

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CD8-expressing cytotoxic T cells (CTLs) are major contributors to the immune defence against viruses and in combating cancer. Their main function is to kill infected or transformed cells by secreting a cocktail of cytotoxic enzymes and this has made them very attractive targets for immunotherapies. In order to eliminate infected targets or cancer cells they need to traffic into inflamed or tumour tissues, which requires adhesion to the endothelium of blood vessels, transmigration into the tissue, migration towards the target cell and eventually adhesion and killing of the target.
We recently identified the kinase WNK1 in an RNAi screen as a negative regulator of integrin-dependent adhesion and a positive regulator of migration of naïve $CD4^+$ T cells. We found that WNK1 regulates adhesion through the small GTPase Rap1 and through its downstream effectors, the kinases OXSR1/STK39. Migration is similarly controlled by OXSR1/STK39, but additionally also through the ion co-transporter SLC12A2. We have now established that the kinase activity of WNK1 is also essential for the efficient degranulation and killing of target cells by cytotoxic T cells, as well as the migration towards and into tumour tissue. While migration and adhesion of the CTLs is controlled by OXSR1/STK39 similar to what we observed in naïve CD4 T cells, killing is not, suggesting that this aspect of WNK1 function is regulated through a novel signalling pathway. WNK1-deficient CTLs show defects in Immune Synapse (IS) formation, actin reorganization within the IS, and the generation of mechanical force, all of which are required for efficient killing. Here we will present our current work which focuses on delineating the molecular mechanisms underlying WNK1 signalling in cytotoxic T cells both *in vitro* and *in vivo*.

P44

Developing a stable setup for two-photon intravital imaging of intestinal immune cells in live mice

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Background

The intestine is one of the most complex organs and a summation of mucosal immunity. It is the hub of interaction between immune cells, commensals, and possibly hazardous foreign materials that have entered the body through food intake. Necessity to obtain intravital images of the interaction between immune cells in the intestine is growing. However, it has been a hurdle to reduce the vigorous movement caused by peristalsis. Here, we established a surgery method to firmly fasten the movement of the intestine for imaging, while maintaining the blood flow in capillaries. Using this intestine imaging setup, we intend to visualize the intestinal microenvironment focusing on immune cell interaction in inflammatory conditions such as colitis.

Methods

Mice were anesthetized and abdominal hair was removed. An incision approximately 2 cm long was made 5 mm under the tip of the sternum. Using a Q-tip, the intestine was carefully taken out of the abdominal cavity. It was opened by a vertical 2 cm long incision and rinsed with PBS to eliminate debris and feces.
3M paper was cut to match the size of a custom-made silicon pedestal and put on top to enhance the fixation and moisturization of the intestine. The intestine was carefully spread on top of the pedestal with the luminal side facing up and held with insect pins. Finally, a custom-made imaging window was screwed into place while firmly pressing the intestine to minimize peristalsis.

Results

We were able to successfully obtain intravital images of the luminal side of the small intestine using a two-photon microscope. We observed the structure of capillaries and migration of neutrophils residing in the intestinal tissue.
We also applied a chronic colitis model to compare the phenotypical outcomes of control and inflamed intestinal tissue, such as leukocyte morphology, migration, and population shift. In addition, we are pursuing to detect the change of intestinal tissue and cell population in the presence of hazardous foreign materials that have entered the body through food intake, which we refer to as '*material X*'.

Conclusions

We have established a method to obtain a stable intravital image of the intestine of live mice using two-photon microscopy. We aim to detect the phenotypical differences in the intestinal tissue and leukocyte migration in control, colitis induced intestines, and colitis intestines exposed to 'material X', and verify whether material X has a more harmful effect on inflamed tissue compared to control conditions.

P45

Hyperplex immunofluorescence as a tool to explore immune cell phenotypes in the tumor microenvironment

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Background

In the past decade, the focus of novel anti-cancer therapies shifted from tumor-intrinsic targets to harnessing and empowering the immune cells to fight against malignant cells (PMID: 34624224). Immune system-targeting approaches have seen increasing clinical success (PMID: 29990692). To further innovate and continue to improve such therapeutical interventions, more detailed knowledge of the tissue-immune cell interaction is needed (PMID: 33811120). Spatial proteomics provides unprecedented and detailed views on immune cell phenotypes while preserving spatial tissue context (PMID: 34811556) and can be insightful for therapy design (PMID: 32466969). Sequential Immunofluorescence (seqIF™) is a novel method, where cycles of antibody-based detection of antigens, imaging, and elution steps are fully automated and integrated on the COMET™ platform. Here, we present how the seqIF™ methodology can be applied to map immune cells across different tumor immune microenvironments (TIME) and underpin their activation phenotypes.

Methods

Formalin-fixed, paraformaldehyde-embedded (FFPE) and frozen section (FS) tissue slides were interrogated with hyperplex panels encompassing main immune cell biomarkers and using off-the-shelf reagents. Hyperplex immunofluorescent protocol was performed with automated staining-imaging COMET™ platform generating ome-tiff images composed of 43 layers: DAPI, 2 autofluorescent, and 40 marker channels for FFPE sample and 35 layers: DAPI, 2 autofluorescent, and 32 marker channels for FS specimens. Image postprocessing was performed with the HORIZON™ Image analysis software.

Results

40 biomarkers were detected on a single tissue slide per automated run and underpinned the heterogeneity in the immune cell distribution across different tumor types present within multiorgan tissue array dataset. Results on both FFPE and FS samples shows excellent tissue preservation through the whole workflow. The imaging enabled the identification of several immune cell phenotypes and provided sufficient resolution to identify the subcellular distribution of detected biomarkers.

Conclusions

SeqIF™ supports in-depth TIME studies that aims identifying cell phenotypes at single-cell resolution, while preserving the spatial context crucial in understanding the complex intercellular interactions. Our data highlights the potential of using such microfluidics-based approaches in the research focusing on TIME.

P46

MPXV may evade effective drug therapy in immunocompromised patients

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Background

The current and still ongoing worldwide outbreak of mpox (former monkeypox) is unprecedented in its size and distribution. As the causative agent, a new monkeypox virus lineage was determined and classified as monkeypox virus (MPXV) clade IIb where mortality is relatively rare. To date, no specific antiviral drugs for the treatment of mpox are available. The drug Tecovirimat was originally developed for the treatment of smallpox, but has shown broad-spectrum activity against other orthopoxviruses including MPXV. Mpox is generally a self-limited disease, and the majority of the infected patients are able to clear the virus and fully recover. In contrast, recent data showed that in patients living with uncontrolled HIV, severe complications are common including secondary infections, necrotizing skin lesions, and increased mortality. However, the mechanisms behind these fulminant mpox disease courses with worse clinical outcomes are poorly understood.

Methods

Here, we performed comprehensive virological and immunohistochemical analyses of an HIV-1-positive patient with low CD4 T cell count, and prolonged mpox infection (> 94 days). MPXV infectivity was assessed by virus isolation on Vero cells. For the first time, a deep skin lesion from a patient was investigated by immunohistochemistry (IHC) and electron microscopy (EM). Findings were verified by in-vitro infection experiments and virus genome sequencing.

Results

Although the patient received two 14-day treatment courses of Tecovirimat, viral titers remained high. Viral titers in the patient's tissue lesion exceeded those of blood testing or mucosal swabs. Staining of different immune cell populations showed that diverse immune cells are recruited to the active zone of the infection, however, are sparse in the necrotic lesion core. Interestingly, macrophages only very rarely co-localized with and did not contribute to eliminating virus-infected cells. Of note, histopathological analyses of infected cell cultures and tissue samples indicate cell-to-cell spreading of MPXV particles that is not targeted by Tecovirimat therapy.

Conclusions

The spreading of intracellular mature MPXV particles might represent an alternative infection pathway that partially undermines Tecovirimat's effect, is unrestricted by immune cell abundance, and thus warrants the development of additional therapeutic options to treat patients at risk of severe mpox disease.

P47

Imaging the activity of neutrophils serine proteases in neutropenia patients using confocal microscopy

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Background

Neutrophils which constitute 50-70% of the leukocytes circulating in the blood, play an essential role in defending the body against pathogens. They are the main component of the innate immune system. Neutrophils recruited to the site of the inflammation can eliminate microorganisms by phagocytosis, in which the pathogen is coated, fuses with lysosomes and is destroyed; degranulation involving the releasing of toxic granules (mainly proteolytic enzymes) or by releasing NETs which are decondensed chromatin riched into neutrophil serine proteases (NSPs) among others. The serine proteases present in the azurophil granules of neutrophils are stored in an active form. These include: NE (neutrophil elastase), CATG (cathepsin G), PR3 (proteinase 3) and the recently discovered NSP4 and GrA (Granzyme A) or GrB (Granzyme B). Neutrophil serine proteases are also externalized in an active form during neutrophil activation at inflammatory sites, thus contributing to the regulation of inflammatory and immune responses. They also play a regulatory role in noninfectious inflammatory diseases and disorders like neutropenia. Neutropenia is a hematological disorder characterized by an abnormally low number of neutrophils. Cyclic neutropenia and severe congenital neutropenia are the two main forms of hereditary neutropenia. Neutrophil serine proteases are extremely important in the context of neutropenia. So far, most attention has been devoted to neutrophil elastase. It is known that NSPS mediate the development of hereditary neutropenia. Both severe congenital neutropenia and cyclic neutropenia are characterized by inhibition of neutrophil maturation at the stage of myelopoiesis. The molecular basis of severe and cyclic neutropenia has been identified as mutations in the *ELANE2* gene encoding neutrophil elastase. Research shows that *ELANE2* mutations interfere with normal intracellular NE transport. This leads to the accumulation of the mutated protein inside the cell and causes neutrophil apoptosis. Purpose of our research is devoted to the creation of the first subcellular map of NSP activity (NE, CatG, PR3, NSP4) in neutrophils from neutropenic patients and healthy donors using confocal microscopy.

Methods

Confocal microscopy. I am preparing glass slides with neutrophils isolating from patients and healthy donors blood circulation. After isolations, neutrophils are staining with ABP (Activity Base Probes) for individual neutrophil serine proteases in purpose of enzyme activity measured. Cells are staining with antibodies also, in purpose of measured the amount of neutrophil serine proteases. Glass slides with neutrophils are imaging using confocal microscopy.

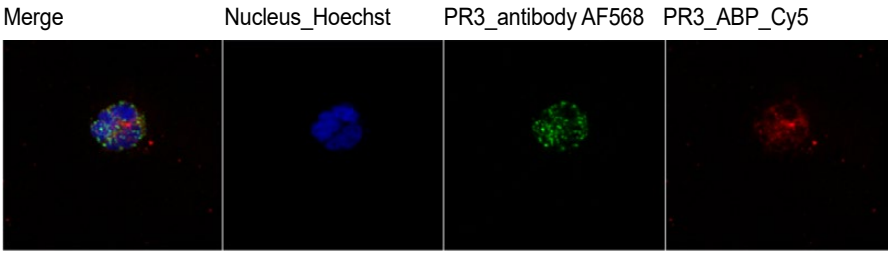

Figure 1. An example of imaging PR3 expression (PR3_antibody AF568) and activity (PR3_ABP_Cy5)

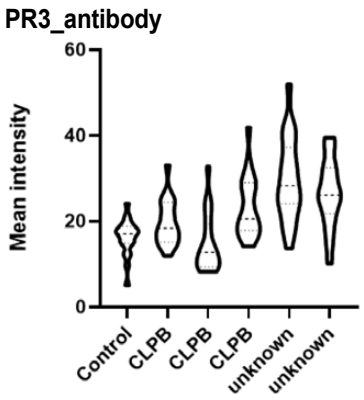

Figure 2. Expression of PR3 in health control control and patients with different mutations

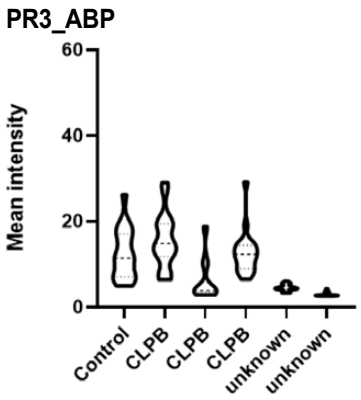

Figure 2. Activity of PR3 in healthy control and patients with different mutations

Conclusions

To sum up, deficiencies of neutrophils in the blood may result from inhibition of their maturation, retention in the bone marrow or defects at the level of the cell leading to its death, i.e. apopotosis. Neutrophils contain specialized granules containing many proteins, including specific neutrophil serine proteases. These proteases are essential for the maturation and functioning of the whole cell, and mutations in the genes that encode them often lead to neutrophil defects. Veryfication of the activity of NSPs in neutropenia patients may help in creating a unique diagnostic test and an effective and safe method of cell therapy based on the editing of altered genes.

P48

CD4+ T cells eradicate IFN-unresponsive melanomas that resist CD8+ T cell therapy

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Background

The cytotoxic properties of CD8+ T cells to eradicate tumours are well established and exploited in standard immunotherapies. Anti-tumour CD4+ T cells can facilitate tumour regression not only directly, but also through several indirect effector mechanisms, but the contribution of CD4+ T cell-mediated tumour control remains incompletely understood. Here, we developed an adoptive cell therapy (ACT) protocol that allows us to investigate the requirements for a successful immunotherapy mediated by CD4+ T cells in direct comparison to the better-understood cytotoxic CD8+ T cells.

Methods & Results

Our ACT protocol consists of preconditioning using cyclophosphamide one day prior to ACT, adenoviral vaccination to prime adoptively transferred TCR-tg CD8+ and CD4+ T cells, followed by innate immune stimulation using poly I:C and CpG. In a transplantable melanoma model, we found that adoptively transferred CD4+ T cells were able to control melanomas as efficiently as CD8+ T cells. Strikingly, CD4+ T cells could still control IFN-unresponsive melanomas deficient in Jak1 expression, while CD8+ T cells failed to control Jak1-KO tumours. To investigate the dynamics of CD4+ and CD8+ T cells in the tumour microenvironment via intravital 2-photon microscopy, we crossed TCR-tg donor mice to eGFP or Venus reporter strains. We found that anti-tumour CD4+ T cells have fundamentally different spatial and dynamic properties when compared to CD8+ T cells. While CD8+ T cells profoundly infiltrated MHC-competent tumour centres and decelerated in proximity to tumour cells, CD4+ T cells were low in abundance and formed local clusters at the tumour invasive margin. In Jak1-KO tumours, CD8+ T cells were not able to infiltrate the tumour centre or form stable interactions with the tumour cells. In contrast, the migratory behaviour of CD4+ T cells did not alter substantially in Jak1-KO tumours. Instead, when utilising the CD11c-YFP reporter strain, we found that CD4+ T cells form long-lasting interactions with CD11c-YFP+ cells in an antigen- and MHC-II-dependent manner.

Conclusions

In summary, our results show fundamental differences in the spatial and temporal behaviour of anti-tumour CD8+ and CD4+. Specifically, CD4+ T cells can recognise tumour-derived antigen bound to MHC-II on CD11c+ cells to subsequently eradicate tumours that evade CD8+ T cell therapy. Exploiting CD4+ T cell effector functions could be used to complement current clinically applied immunotherapy strategies.

P49

Uncovering the spatiotemporal activity of a T-cell Dependent Bispecific antibody in lymphoma bearing mice using intravital imaging

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Beatrice Corre, Zacarias Garcia, Fabrice Lemaître, Philippe Bousso

T-cell Dependent Bispecific antibodies (TDBs) are promising off-the-shelf T cell engaging therapies, that redirect the cytotoxic activity of T cells towards tumor antigens in a TCR/MHC-independent fashion.
CD20-TDB, particularly, is emerging as a major therapeutic option for B cell lymphoma patients, yet the kinetics and cell dynamics that underlie its mode of action are currently unknown.
We developed a versatile murine model relying on the transduction of the human target molecules of CD20-TDB, to decipher how this treatment activates and enhances cytotoxic T cells to kill B lymphoma cells.
Taking advantage of the single-cell resolution offered by 2-photon microcopy, we imaged interactions between T cells and tumor cells in the bone marrow of lymphoma bearing mice. We uncover that activation occurs rapidly after a unique injection, reshaping T cells' behavior and interactions with lymphoma cells. Importantly,
we show that the killing capacities of T cells are markedly increased within minutes after a unique injection of CD20-TDB. Characterizing how this killing rate evolves over time, and determining the factors which sustain or hinder T cells' cytotoxicity, appears essential to determine CD20-TDB's mode of action.
Together, our preliminary results and future work should provide key insights on how CD20-TDB boosts tumor killing by T cells, a major requirement to further optimize this treatment and determine the patients who will most benefit from it.

P50

Investigation of β1 integrin dependency in the process of neutrophil migration of the inflamed and damaged adipose tissue

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Background

Adipose tissue harbors a wide range of immune cells that can regulate acute and chronic immune response by adipokines and cytokines. Most of studies have mainly investigated chronic inflammation in adipose tissue such as obesity. However, little has been known for neutrophil migration during acute and systemic inflammation in adipose tissue. Lipopolysaccharide (LPS) causes systemic inflammatory environment like sepsis. In response to LPS stimulation, neutrophils infiltrate to adipose tissue and other organs. Integrin-ligand binding is one of the critical factors in immune cell migration. Especially, β1 integrin is known to be involved in the migration of immune cells in interstitial tissues. Therefore, we are investigating the role of adhesion molecules, β1 integrins in neutrophil migration on inflammatory adipose tissues.

Methods

We conducted flow cytometry and two-photon intravital microscopy (TPIVM) to verify neutrophil population in adipose tissue. For inducing systemic inflammation, E. coli LPS was intraperitoneally injected to male mice. To measure the number of neutrophils, epididymal white adipose tissue (eWAT) was isolated from mice and stromal vascular fraction was collected. Also, we performed in vivo imaging in C57BL/6 and LysMGFP mice using TPIVM. Exposed eWAT was immersed for staining adipocytes and blocking integrin.

Results

Flow cytometry results showed that the neutrophil population in eWAT was significantly increased 24 h after E. coli LPS injection compared to control. Using TPIVM, we confirmed that neutrophils were infiltrated more in the LPS-treated group than those in the control. When laser burning was given to a single adipocyte, neutrophils were vigorously induced and then accumulated into the damage spot of the inflamed eWAT. Through cell tracking analysis, displacement rate and meandering index have no difference between control and LPS-treated group. However, the velocity was more significantly decreased in LPS-treated group than the control. This result indicated that activated neutrophils become strongly adhesive with adhesion molecules on eWAT.

Conclusions

To explore adhesion molecules dependency of neutrophil migration on the physically damaged tissue, migrating neutrophils toward laser pulse-damage site in the adipose tissue will be analyzed using TPIVM in real time manner. Through topical administration of integrin blocking antibody, we pursue to reduce the excessive accumulation of neutrophils by inhibiting integrin-ligand interaction.

P51

Antibody-mediated immunity during nasal vaccination

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Background

Nasal vaccination is a promising approach for generating protection against airway pathogens, with nasal-associated lymphoid tissue (NALT) playing a crucial role in initiating immune responses. However, the unique structural features of NALT and the reasons for the reduced effectiveness of primary vaccination compared to prime-boost vaccination remain unclear. Understanding the complex interactions between the various cell populations in NALT may provide insights into developing more effective nasal vaccines.

Methods

In this study, we developed a novel nasal vaccine mouse model combine with adaptively transferring antigen-specific B cells and T cells. By combining 2-photon and light-sheet microscope imaging, single-cell RNA sequencing and flow cytometry analysis, we examined the immune response in the NALT following nasal vaccination with NP-OVA. Specifically, we investigated the dynamics of antigen-specific responses, B-T cell interaction, and plasma cell maintenance during nasal vaccination.

Results

Our study found that nasal vaccination leads to extensive B cell expansion in the subepithelial dome (SED) niche, whereas T cells originating from inter-follicular regions. T-B interactions were observed in the SED early during the response, and B cell clonal expansion in this niche was MHC-II-dependent. Then, we found that antigen-specific B cells could not respond without the presence of high levels of naive T cell precursors or initial pre-expansion of endogenous T cells. Moreover, antigen-specific effector B cells were detected in olfactory epithelium after intranasal prime-boost vaccination.

Conclusions

Our study revealed that nasal vaccination induces MHC-II-dependent B cell expansion in the SED niche, and T cells originated from inter-follicular regions, with T-B interactions observed in the SED early during the response. Effective nasal vaccination induces extensive B cell expansion that depends on sufficient cognate T cell help within the SED niche. Additionally, intranasal prime-boost vaccination drives antigen-specific B cells to the olfactory epithelium, thereby protecting the upper airway and brain. Our study provides new insights into the mechanisms underlying the immune response to nasal vaccination, highlighting the importance of T-B cell interactions and the SED niche in driving protective immunity for the upper airway and brain. These findings may have important implications for the development of more effective vaccines against respiratory infections, particularly in the context of emerging viral pathogens that affect the respiratory system.

P52

Peptide-MHC-I-targeting chimeric antigen receptor (CAR) and T cell fusion constructs (TRuC) T cells form T cell receptor-like immune synapse morphology and cytotoxicity

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Background

Chimeric antigen receptors (CARs) and T cell fusion constructs (TRuCs) are synthetic receptors, engineered into T cells to harness the cytotoxic functions of T cells towards to any tumour antigen of choice. There has been increasing interest in understanding how the receptor design could influence killing kinetics, persistence, and efficacy to improve and develop novel immunotherapies.

Methods

To evaluate the effectiveness our CARs and TRuCs, we studied the morphology of the immune synapse, the junction between the T cell and tumour cell, which consists of highly organised positioning of cytoskeletal and signalling components required for effective T cell cytotoxicity. Using laser scanning confocal microscopy and lattice lightsheet microscopy, we were able to visualise and interrogate the morphology of the T cell immune synapse and perform time lapse studies into T cell killing kinetics, measuring the time between T cell contact, signalling, degranulation, and target cell death.

We generated synthetic receptors against known tumour antigens in various formats, including surface antigen-targeting TRuCs and second-generation peptide-MHC-I (pMHC)-targeting and surface antigen-targeting CARs, to compare the morphology and functionality of CARs and TRuCs against the native T cell receptor (TCR).

Results

We found that both pMHC-targeting CARs (Wang et al, 2021, Biomedicines) and TRuCs form a morphologically TCR-like immune synapse and TCR-like cytotoxicity, contrasting with CARs targeting surface antigen HER2 (Davenport et al, 2016, PNAS). Interestingly, despite other groups reporting transcriptional and functional differences between receptor design and choice of co-stimulation (Boroughs et al, 2020), mesothelin-specific CAR T cells with either 41-BB or CD28 co-stimulation domains both showed similar TCR-like immune synapse morphology and TCR-like killing kinetics, both effectively killing antigen-expressing tumour cells.

Conclusions

Our findings imply that both the synthetic receptor design and the tumour antigen play a role in the formation of the immune synapse, which directly contributes to T cell cytotoxicity. Further work is required to understand how the combination of the receptor and tumour antigen influences immune synapse formation and T cell killing kinetics, so that we could further optimise and improve our synthetic receptor designs to create more effective immunotherapies.

P53

The power of intravital two-photon imaging of Chronic Cranial Windows: insights into T cell dynamics and the influence of the tumour microenvironment in mouse models of glioma.

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Background

Chimeric Antigen Receptor (CAR) T cell therapy involves re-engineering patient-derived T cells to redirect T cell cytotoxicity against tumour cells. Whilst CAR T cell therapy has demonstrated remarkable success in treating haematological malignancies, the same success has not yet been recapitulated in solid tumours, including brain tumours. To better optimize the design and delivery of immunotherapies, it is crucial to understand the dynamics of CAR T cells in vivo and the influence of other cellular players present in the tumour microenvironment.

Methods

We have established a unique, chronic cranial window implant, which provides a window into the brain of immunocompetent mouse model of glioma (Mulazzani et al, 2019, PNAS). In combination with two-photon microscopy, this allows us to visualize tumour cell growth intravitally, longitudinally over the entire tumour course. We can examine the extent of T cell infiltration, accumulation, directionality (movement towards and away from tumour), velocity and persistence across time in the same mouse.

Results

We found that antigen-specific CAR T cells reached the brain of glioma-bearing immunocompetent mice only 48 hours after one intravenous dose of CAR T cells. Whilst the CAR T cells continued to show accumulation in the brain two weeks following injection, the CAR T cells did not show migration towards or away from tumour; instead, they travelled along tumour border. Despite our *in vitro* T cell killing assays demonstrating CAR T cell effectiveness, they were unable to clear the tumour *in vivo* and the mice all eventually succumbed to disease. This suggests a possibility of other cellular players, such as microglia, within the tumour microenvironment hindering T cells cytotoxicity, emphasising the need for combination therapy approaches.

Conclusions

In conclusion, this cutting-edge *in vivo* intravital imaging technique allows for real time and longitudinal insight into the cellular kinetics of CAR T cell therapy in brain tumours, allows us to gain new insights into the complex interactions between CAR T cells and other immune cells and tumour cells in the brain microenvironment, which can ultimately inform the development of more effective immunotherapies for brain tumours.

P54

Imaging of Legumain in a Syngraft Mouse Model by Positron Emission Tomography

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Background

Anti-cancer treatment has undergone a paradigm shift with the advent of cancer immunotherapy. Despite promising achievements, these therapies are restricted to a limited range of cancer types and patients. The tumor microenvironment is a potential source of promising therapeutic targets, in addition to the well-established immune checkpoints. The cysteine endopeptidase legumain is overexpressed in tumor-associated macrophages and promotes cancer metastasis. Our goal is to develop PET tracers to support research and development towards legumain-targeting anti-cancer therapies.

Methods

The affinity of the tracer RO-01 towards legumain was assessed using proteolytic assays. The precursor was radiolabeled via ^{11}C O-alkylation. The binding specificity of [^{11}C]RO-01 was evaluated by autoradiography on CT-26 mouse tumor tissue slices. Legumain fluorescence microscopy was performed to compare with autoradiography. PET scans were conducted with CT-26 tumor-bearing mice to determine the in vivo tracer distribution. The results obtained from the scans were validated by analyzing the dissected tissues. Radiometabolites were evaluated using column-switch RP-HPLC.

Results

ERO-01 demonstrated a binding affinity (Ki) of 5.4 nM and 6.4 nM to mouse and human legumain, respectively. [^{11}C]RO-01 was synthesized with a radiochemical purity of > 99 % and a molar activity of 203 GBq/µmol. Autoradiography showed heterogeneous tracer accumulation in the tumor slices. However, the accumulation was not blocked by excess unlabeled RO-01. Fluorescence microscopy confirmed heterogeneous distribution of legumain within the tumor tissue. In PET, [^{11}C]RO-01 accumulated in the tumor periphery. Biodistribution analysis after dissection indicated no significant difference between baseline and blocking conditions in tumors. The tumor uptake of [^{11}C]RO-01 was significantly higher than in muscle, and blocking had an effect in organs with high legumain expression. At 45 minutes post-injection, plasma predominantly contained parent tracer.

Conclusions

We have successfully developed a stable, high-affinity, high-potency legumain PET tracer. A blocking effect with RO-01 in kidney and spleen suggests high specificity. We are currently investigating why tumor accumulation was not blocked by excess RO-01.

P55

Exocrine gland-resident memory CD8+ T cells use mechanosensing for tissue surveillance

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Background

Tissue-resident memory $CD8^+$ T cells (TRM) constitutively scan stromal cells in their tissue of residence, such as skin, gut or visceral organs, to intercept microbial spread. While chemokines and integrin ligands produced at epithelial barriers are critical mediators of this process, their elevated constitutive expression in non-barrier organs might lead to excessive influx of immune cells.

Methods

Flow cytometry, Immunofluorescence, 2PM image acquisition, Under agarose and confinement chamber assays, 3D collagen matrix migration, CRISPR/Cas9-medited MYH9 deletion in T cells, Nanostring, MCMV rechallenge

Results

We found that exocrine gland T_{RM} are programmed for autonomous tissue scanning in the absence of any chemoattractant or adhesion receptor engagement. The signals eliciting this non-canonical motility mode and its relevance for organ surveillance have remained unknown. Here, we report that exocrine gland T_{RM} autonomously generated retrograde F-actin flow for locomotion, accompanied by high myosin IIA-dependent cortical contractility and leading edge bleb formation. The distinctive mode of exocrine gland T_{RM} locomotion was triggered by sensing physical confinement of its microenvironment by the nucleus. Pharmacological blockade of mechanosensensing mediated by nuclear deformation disrupted autonomous motility and target cell identification by exocrine gland T_{RM}. .

Conclusions

Mechanosensing of physical confinement suffices to elicit homeostatic T cell surveillance of exocrine glands, and acts to complement chemosensing-mediated migration in non-inflamed organs.

P56

Enhancing CD8+ T cell activation and infiltration into melanoma metastasis with engineered Escherichia coli trained CD4+ T cells: a two-photon microscopy study

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Background

We have shown that Listeria monocytogenes capture by conventional CD4+ T cells train them to become antigen presenting cells (APC), which activate very efficiently CD8+ T antitumor responses. To improve antitumor responses and design a safer immunotherapy, we engineered a non-pathogenic Escherichia coli strain (eng-E. *coli*). Eng-E. coli BacT elicit higher CD8+ T cell activation than L.
monocytogenes BacT, as well as low PD-1 and CTLA-4 expression levels, increased proinflammatory cytokine secretion and augmented metabolic parameters, desired features for effective anti-tumor responses.
Moreover, melanoma growth in vivo is significantly slower when using eng-E. *coli* BacT as ATC in comparison to *L. monocytogenes* BacT, and the tumor immune infiltrate analyzed by spectral flow cytometry is different comparing both therapies.

Methods

In this context, we used intravital two-photon microscopy to assess the efficient T-cell targeting, infiltration and activation of the different CD8+ T cell responses activated by eng-*E. coli* or L. *monocytogenes* BacT, following the subsequent methodology: first, we injected B78-OVA melanoma cells into the footpad of immunocompetent mice. Then, when the metastasis appeared in the popliteal lymph node (pLN), we used ex vivo activated CD8+ T cells with eng-*E. coli* or *L. monocytogenes* BacT as therapy, in comparison to not activated CD8+ T cells.

Results

Thus, we have evaluated the dynamics of the tumor CD8+ T cell infiltrate by intravital microscopy following therapy with ex vivo activated CD8+ T cells with eng-E. coli or *L. monocytogenes* BacT, to deeply understand what do they have in common to be so efficient in tumor elimination and in which aspects do they differ to be the eng-*E. coli* BacT activated CD8+ T cells the most efficient therapy.

Conclusions

We have assessed the differential CD8+ T cell dynamics, morphology, mobility and trafficking in B78-OVA melanoma metastasis in the pLN. This work has revealed novel morpho-dynamic characteristics on cytotoxic CD8+ T cells behavior depending on the type of BacT functioning as APC, emphasizing that it may be the case that the phenotype of the APC activating CD8+ T responses could profoundly influence their fate and function.

P57

Mast cells trap and cannibalize swarming neutrophils for metabolic and inflammatory fueling

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Background

Degranulating mast cells (MCs) release inflammatory mediators, including chemokines and chemoattractants, which attract immune cells to sites of anaphylaxis. Neutrophils can initiate self-amplifying swarming responses via intercellular communication through the lipid leukotriene B4 (LTB4). However, the dynamics of neutrophils during anaphylaxis and the direct interaction with MC in tissues has been poorly investigated. Further, the impact of neutrophils on exhausted MCs after anaphylactic degranulation is hardly characterized. Here we investigated how MC activation influences neutrophil dynamics during anaphylaxis and analyzed whether and how neutrophils modify the recovery of exhausted MCs.

Methods

We performed two-photon intravital microscopy in mice, confocal live cell imaging and multiple static imaging methods to characterize interaction dynamics between degranulating primary MCs and neutrophils. We used Ribotaq precipitation, RNAseq and mass spectrometry analysis of co-cultivated cells to identify the impact of neutrophils on recovering MCs. Finally, we proofed neutrophil impact on mast cell functionality and survival.

Results

We show that degranulating MCs release LTB4 and exploit this attractant by re-directing neutrophils and inducing their swarm formation in vivo. Unexpectedly, neutrophil cluster formation around MCs results in the trapping of living neutrophils inside MC vacuoles in vivo and in vitro. Thus, we identify a novel cell-in-cell structure between MCs and neutrophils, which we term “Mast Cell Intracellular Trap” (MIT). Trapped neutrophils undergo cell death and fuel MC metabolism. This leads to several benefits for MITs: (1) they can be more efficiently re-stimulated than MCs, (2) they show improved survival under nutrient limitation, and (3) MITs store neutrophil DNA and effector molecules like myeloperoxidase. This stored neutrophil material can be released after re-stimulation, modifying the inflammatory profile of MCs. A process that we call “nexocytosis”.

Conclusions

The invasion of a living cell into the cytoplasm of another cell (entosis) has been described for several cell types.
We here show that MCs undergo a previously unappreciated entosis-like process, which promotes their regeneration, functionality and inflammatory potential. In addition, we define a novel role of neutrophils, which supply nutrients and inflammatory molecules to recovering MCs.

P58

Determinants of CD8+ T cell behavior within hepatocellular carcinoma

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CD8+ T cells play a crucial role in controlling liver tumours, such as hepatocellular carcinoma (HCC) however we have only limited knowledge of the precise dynamics of their interactions with hepatic parenchymal and non-parenchymal cells at the single-cell level.

Previous work from our laboratory, demonstrated that in the context of HBV-expressing hepatocytes circulating effector CD8+ T cells (T_{eff}) perform their immune surveillance function recognizing the antigen and kill virus-expressing hepatocytes extending cytoplasmic protrusions through endothelial fenestrations while still within liver sinusoids. Here we dissected whether similar or different mechanisms govern the capacity of Teff to home, migrate, recognize the antigen, and exert effector function within HCC.

The first effort to dissect the project was the establishment of a new murine model of spontaneous HCC in which just the transformed hepatocytes express a nominal antigen, the oncogene SV40 large T antigen (TAg), and a fluorescent protein. We were able to obtain mice that develop spontaneous HCC lesions, highly proliferating and spread in a normal liver parenchyma. After in vitro effector differentiation and adoptive transfer of TAg-specific Teff in tumor-bearing mice, we observed that just some mice respond to the cytotoxic activity of the transferred cells, eliminating partially or completely the tumor, while in other mice the adoptive transferred cells have no beneficial effect on the tumor elimination. Thus, using a mathematical approach, we managed to pick the lesion volume as the fundamental parameter to predict the fate of each single HCC lesion: we called “responders” (R) the HCC lesions that are responsive to the cytotoxic activity of the TAg-specific Teff, with a single HCC lesion volume <10 mm^3, while we called “non-responders” (NR) the HCC lesions that are not responding to the cellular therapy and they have a single HCC lesion volume > 100mm^3.

P59

Impact of tumor associated macrophages on Extracellular matrix biophysical properties and associated immune cell infiltration

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The extracellular matrix constitutes the structural scaffold for organs and tissues and provides key physical and biochemical cues to cells. While the profound remodeling of the ECM during tumorigenesis has been long reported, its effect on anti-tumoral immune responses is still not fully understood. In this work, we wish to investigate first how macrophages, key immune drivers of tumorigenesis, modulate the biophysical properties of the tumoral ECM and secondly how those modulations impact on the anti-tumoral response. To this end, we are developing an interdisciplinary approach at the frontier of immunology, cell biology and physics. Extensive characterization of the tumoral ECM local topography is performed on colon-carcinoma tumors (MC38) transplanted in mice in which macrophages can be depleted at different time points of tumorigenesis. This allows us to get dynamic understanding of fiber remodeling during tumorigenesis and assess the role of macrophages in this process. To understand specifically how ECM is remodeled upon macrophage depletion and correlate those changes with ECM topography and immune infiltration, we developed a multi-omics approach, using scRNAseq and spatial transcriptomic combined with whole tumor confocal and SHG-collagen imaging. Our results show that the tumoral extracellular matrix is modified both at the tumor edge and within the tumor core upon macrophage depletion. The collagen capsula appears thickened and disorganized, while fiber-rich areas are observed more frequently within the tumor core. Both the capsula and those fiber-rich areas arbor a massive accumulation of T lymphocytes and neutrophils, also observed in scRNAseq. We are now using artificial intelligence and computational strategies to better characterize the collagen organization and assessing more ECM properties such as stiffness by AFM and composition by mass spec proteomics to uncover the functional link between our observations. We think that our strategy will foster deeper understanding of the mechanisms involved in matrix remodeling by macrophages and its involvement in shaping the tumor microenvironment. We foresee that our project will bring important fundamental knowledge on the interaction of immune cells and the extracellular matrix in the tumor and will contribute to the emergence of the nascent field of immunophysics.

P60

Role of autophagy in T cell homeostasis

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Background

Antigen-inexperienced, naïve T lymphocytes may remain in a resting state for very long times. Encounter with their cognate antigen triggers their activation and clonal *expansion*, which consists of increase in cell size, proliferation, enhanced protein production and secretion of signaling molecules. This is followed by a *contraction phase*, where cells stop to proliferate and reduce their size. They may eventually re-gain a resting (memory) state.

The Endoplasmic Reticulum (ER) and mitochondria are major organelles of eukaryotic cells. The ER is site of proteins, lipids, oligosaccharides synthesis. Mitochondria produce most of the energy required for cellular functions. Activation of naive T cells elicits a transient metabolic boost characterized by ER and mitochondria expansion to support the increase in biosynthetic and bioenergetic demand. Less is known on catabolic programs that may intervene to reduce ER and mitochondrial mass at the end of the activation phase, when T cells stop proliferating, reduce their size and may undergo transition to a memory status. Notably, lack of general autophagy in mice reduces the quality and quantity of memory T cells after infection. However, what type of autophagy intervenes during these processes and the modality of intervention remain to be established.

Methods and Results

Here, we employed imaging techniques, including confocal microscopy and transmission electron microscopy, and developed automated deep-learning-based image analysis methods to assess the involvement of autophagic pathways in human primary T cell maintenance and activation. The data obtained show high levels of constitutive ER-phagy and mito-phagy in resting naive and memory T cells, reveal that ER-phagy and mito-phagy are transiently shut down immediately after cell activation, and that ER-phagy but not mito-phagy is eventually resumed during the contraction phase.

Results

Our findings suggest that regulated lysosomal clearance of organelles may play an important role in T cell biology.

P61

Assessing spatial distribution of dendritic cells in the TME upon FAP-CD40 treatment using 3D multiplexed imaging

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Background

Therapies targeting the activation and recruitment of dendritic cells (DCs) to the tumor microenvironment (TME) are considered as highly promising approaches to improve anti-tumor immune responses due to the ability of DCs to efficiently cross-present tumor antigens and prime CD8 T cells. While infiltration of the TME by cross-presenting DCs is associated with improved prognosis, the spatial distribution of DCs within the TME and their interaction with other immune cells remains poorly understood. Here, we investigate the spatial localization and activation state of DCs upon tumor-targeted CD40 stimulation in the syngeneic tumor models KPC and EMT6 to better understand the different therapeutic responses to CD40 agonism observed in preclinical studies.

Methods

FAP-CD40, a tumor-targeted CD40 agonist antibody, triggering the activation of DCs specifically in the tumor, was injected into mice bearing KPC (sc) and EMT6 (imfp) tumors. 3D immune phenotyping (3DIP), coupling multiplexed confocal imaging with a quantitative spatial analysis pipeline, was used to analyze DC distribution after treatment. This technology enables the visualization and quantification of up to 12 markers within 70 µm thick tumor sections, facilitating the characterization of complex cell subsets and intercellular interactions directly within intact tumor tissues.

Results

DC1s mainly accumulate at the tumor edge in the excluded tumor models KPC and EMT6 at baseline. Moreover, the majority of DC1s display an immature phenotype, as characterized by their plump shape. Early upon therapy injection, DC1s showed an elongated and stellate cell morphology in both tumor models, indicative of activation in both settings. However, while CD40 agonism leads to a decrease of intratumoral DC1s and increased infiltration of stem-like CD8 T cells to the tumor core in the KPC model, DC1s remained in the EMT6 tumor, specifically towards the edge, with few stem-like CD8 T cells present within the tissue.

Conclusions

Our results suggest that tumor-targeted CD40 agonism can lead to DC1 activation and egress from the tumor to the draining lymph node, resulting in enhanced T cell priming and increased efficacy. In non-responding tumors (e.g. EMT6), although activated, DC1s might be endowed with reduced migratory capacities due to possible inhibitory factors expressed in the TME. Using 3DIP and other spatial technologies, different components of the TME such as the stroma will be further investigated.

P62

Chemokine producers reveal tissue-specific neutrophil niches

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Background

Once regarded as unsophisticated killing machines, neutrophils today are recognized for contributing to a variety of fascinating non-canonical functions, associated with tissue-specific signatures. Currently, the functional spectrum of neutrophils, their tissue-specific interaction partners, as well as the mechanisms behind their reprogramming are poorly understood. In this project, we embarked on an organism-wide expedition to illuminate tissue-specific neutrophil niches that instruct immune functions.

Methods

We generated a novel CXCL1 (*Cxcl1*^{tdTomato}) and CXCL12 (*Cxcl12*^{GFP}) double-reporter mouse to map two potent neutrophil chemoattractants. Taking advantage of tissue-clearing techniques and confocal imaging, we conducted a thorough spatial characterization of CXCL1^{+} and CXCL12^{+} protein expression as well as neutrophil distribution, across 12 tissues. We complemented these efforts with flow cytometry and transcriptomics to identify the cellular sources of each chemokine and their respective molecular programs. To distinguish in vivo interaction partners of neutrophils, we generated a neutrophil-specific lipoSoluble mCherry reporter (*Neutro*^{sherry}) mouse line (inspired by Ombrato, *Nat Protocols 2021*).

Results

We determined that similar types of structural cells, including endothelial cells, pericytes, and fibroblasts, as well as certain specialized parenchymal cells i.e., keratinocytes, hepatocytes, and adipocytes, were responsible for homeostatic CXCL1 and CXCL12 production. The distribution of the cells producing either chemokine appeared to follow distinct anatomical programs that differed from tissue to tissue. Neutrophils displayed a clear predilection towards CXCL1 in the skin and appeared to exhibit an inflammatory program, unlike reparative lung neutrophils which resided preferentially in CXCL12+ niches. We will next evaluate direct interaction partners of neutrophils in tissues using *Neutro*^{sherry} mice and in parallel, study the functional role of neutrophil niches in systemic and local perturbations.

Conclusions

While the project is currently in its infancy, we have a clear roadmap to identify relevant functional programs associated with neutrophil niches and whether their remodeling underlies or synchronizes with certain diseases, such as obesity, or impaired wound healing. By tracking normal or aberrant immune niches, we hope to gain invaluable insights into innate immune reprogramming and unveil functional hallmarks of inflammatory states.

P63

The role of monocytes and macrophages during pyelonephritis

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Background

Pyelonephritis (PN) is an infection of the kidneys occurring as a complication of urinary tract infection. The main pathogen leading to the disease is uropathogenic *Escherichia coli* (UPEC).

While many patients can handle the infection well and recover, severe and recurring infections are a common problem, especially in elderly or immunodeficient patients. During PN, macrophages in the kidneys and recruited neutrophils act as a first line of defense. After infection, macrophages can be replenished by clonal expansion or by infiltrating monocytes.

The role of monocytes during acute PN and on recovery is still unknown. We aim to elucidate the role of monocytes and study how macrophages are replenished after infection.

Methods

To determine the role of monocytes, we use CCR2 knock-out mice. 2-photon intravital microscopy allows us to investigate monocyte infiltration and recovery process in PN as well as the response to recurring infections. $CCR2^{+/RFP}$/ $CX3CR1^{+/GFP}$ mouse model that labels the classical proinflammatory ($CCR2^{hi}/CX3CR1^{low}$) monocytes red and alternatively activated ($CCR2^{low}/CX3CR1^{hi}$) monocytes green will be used in combination with the imaging window to image the same field of view in the kidney over time. This will address the question if infiltrating red proinflammatory monocytes develop into alternatively activated green ones and eventually differentiate into macrophages. We also want to determine the differences of the innate immune response of a first infection compared to recurring PN. We will perform re-infections in the mouse model and investigate if the innate immune cells show effects of trained immunity..

Results

Total CFU number/kidney as well as neutrophil number was reduced in monocyte-deficient mice at 6- and 24-hour post-infection. With 2-photon intravital microscopy we observed a tendency towards higher average velocity of classical proinflammatory monocytes at 24-hour postinfection.

We also saw some yellow cells, suggesting that there was a class switch between monocyte subtypes.

Conclusions

Monocytes have detrimental role in the course of PN. To investigate potential mechanism that mediate improved clearance in monocyte-deficient mice, we want to analyze cytokine production in the kidney. Perhaps lack of monocytes protects against hypercytokinemia, the immune reaction when the organism releases too many cytokines into the blood too quickly, worsening the disease outcome.

P64

New immune-interacting lymphatic endothelial cell subtype as a bridge between lymphatic and immune response in lymphatic malformation pathology

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Background

The lymphatic vasculature of the skin has an essential role in the drainage of interstitial fluid and trafficking of immune cells to the local lymph nodes. These important functions require specific vascular architecture of blunt-ended initial lymphatic capillaries, which drain into larger collecting vessels.

Methods

To strictly control the number of antigen-presenting DCs and antigen-specific $CD8^+$ T cells, we employed a reductionist model whereby recipient mice received 10,000 ovalbumin-specific $CD8^+$ T (OT-I) cells one day before subcutaneous immunization with bone marrow-derived DCs (BMDCs) loaded with OT-I peptide. Using light sheet fluorescence microscopy, we determined the ratio of BMDC to OT-I cell in reactive lymph node (LN) during the first days of a burgeoning $CD8^+$ T cell response. We then correlated the BMDC/OT-I ratio at the early phase to OT-I cell number 7 days after immunization.

Results

Using single cell RNA sequencing, we identified dermal lymphatic endothelial cell (LEC) hierarchy that recapitulates the lymphatic vascular architecture, and defined a previously unknown, molecularly distinct population within capillary terminals as a putative immune-interacting LEC (iLEC) subtype. We further found that in a mouse model of lymphatic malformation (LM), iLEC population is selectively expanded and acts as a driver of disease pathology by interacting with the immune cells. iLECs from mutant mice promote the recruitment of myeloid cells, which in turn produce lymphangiogenic factors and sustain pathological lymphangiogenesis. Nonsteroidal anti-inflammatory therapy limited pathological lymphatic vessel growth, providing evidence for the importance of inflammation response in LM.

Conclusions

The identification of iLECs and their role in LM pathogenesis indicate that peripheral lymphatic vessels not only respond to inflammation but also actively orchestrate the immune response.

P65

Super-deep intravital imaging: breaking the fundamental penetration depth limit for biological discovery

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Background

Intravital two-photon microscopy (IV-TPM) enables deep tissue imaging at high temporospatial resolution in live animals by exploiting a natural optical window in the NIR wavelength range to which biological tissues are 'transparent'. However, certain tissues, such as the thick mineralised bone cortex, pose additional challenges due to the geometry and intrinsic optical properties of the tissue. These impediments to efficient deep-tissue two-photon excitation include the reflection, absorption, scattering, dispersion and polarisation of light as it travels across media with different refractive indices. These impediments are unique to the tissues and pose challenges to efficient deep-tissue intravital imaging of immune compartments in the skin, lymph node, brain, spleen, liver and bone.

Methods

We developed and implemented the first commercial-ready IV-TPM with Raster Adaptive Optics (RAO) module embedded in the light path of a customised Olympus FVMPE-RS multiphoton microscope. This Plug and Play system that can be operated without any need for advanced optics training or experience. The user-friendly GUI can be used to measure and correct for image distortion from aberration and scattering using standardised metrics for AO compensation and image quality improvements.

Results

We develop local and global solutions for AO imaging across different tissues which will be available via an open-source repository. We benchmark the RAO against conventional IV-TPM and show its superior performance for 'super-deep' intravital imaging.

Conclusions

RAO-enabled IV-TPM may achieve super-deep intravital imaging and provide opportunities for biological discovery at depths previously beyond the reach of conventional IV-TPM.

P66

Immunosuppressive microenvironment supports premalignant progression in murine squamous cell carcinoma

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Background

Lung squamous cell carcinoma (LSCC), the second most prevalent subtype of LC, accounts for 22% of cases. It is strongly associated with smoking, with approximately 90% of cases attributable to smoke exposure. Despite this strong association, not all smokers will develop LC. From this, two key questions arise: which patients will develop the lesions preceding LSCC, and among those, which will progress to carcinoma? Stratification of at-risk patients for diversion into prevention and intervention strategies, such as increased monitoring and chemical interception, remains an urgent unmet need, and a greater understanding of the factors contributing to LSCC premalignant lesion (PMLs) progression is critical in addressing this. Previous evidence suggests a role for the immune system in the development of preinvasive disease, and we hypothesize that immune differences between at-risk smokers may also play a role in which patients progress to frank LSCC and which do not.

Methods

Using the carcinogen N-Nitrosotris-(2-chloroethyl)urea (NTCU) murine model, we sought to explore the immune contexture of LSCC premalignancy in two mouse strains with differing susceptibility to NTCUinduced dysplasia. We performed immunophenotyping via imaging mass cytometry (IMC) over the course of disease initiation and progression to carcinoma.

Results

The IMC data has revealed that even at the same histological grade, immune differences existed between the NTCU-sensitive (A/J) and intermediate strain (SWR/J), where there is a 2 fold increase in B cells, 1.5 fold increase in CD8+ T cells, and 5 fold less neutrophils as compared to the sensitive strain. These cell types are established to contribute to LC prognosis, with B cells and CD8+ T cells associating with better survival and inflammatory populations such as neutrophils associating with poorer prognosis. These data suggest that an immunosuppressive and inflammatory microenvironment in the NTCU-sensitive strain may contribute to the differential susceptibility to premalignant lesion development in the two strains.

Conclusions

Characterization of the immune differences underpinning the strain-dependent susceptibility to NTCUinduced dysplasia will lead to an enhanced understanding of the differences between patients with LSCC PMLs that are likely to progress to carcinoma and those that will not, with the ultimate goal of developing and deploying immunomodulatory interventions to ameliorate progression to LSCC and ultimately reduce LC mortality.

P67

Visualization of immune responses in the liver premetastatic niche prior to tumor cell arrival

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Background

Liver metastases result from the blood spread of cells from a primary turnar to the liver, which has a rich blood supply, and are the main cause behind cancer related death, frequently targeted by many different cancer types, as colon cancer arnong others. The liver is a highly organized organ, specifically in terms of immune tissue organization with many organ-specific features. We decided to explore immune celi dynarnics within the liver in prirnary turnar bearing rnice, prior to the spleen injection of turnar cells, in arder to later visualize if immunotherapy regimes alter premetastatic niche patrolling and immunity.

Methods

We performed intravital microscopy in severa! immune-cell reporter mice and tissue immunotluorescence in mice bearing subcutaneous MC38 tumors. Time lapse video analysis and semi-automatic tracking allowed us to characterize subset specific dynamics in the liver, while the analysis of tissues immunotluorescence endows us to study the immune-zonation of myeloid-lineage cells in the same context.

Results

T celi immunosurveillance of the liver is mainly performed by patrolling T and CD 11 e^+ cells, whereas NK cells showed more limited patrolling capacities in turnar bearing mice. Among T cells, $CD4^+$ T cells identified with tluorescent antibodies showed more extensive patrolling and scanning of liver sinusoids than $CD8^+$ T cells. Ptx treatment, which affects the chemokine signaling, altered the proportion of T cells performing patrolling and resulted in less T cells arrested in the liver sinusoids. Although T cells were frequently observed scanning the surface of resident $F4/80^+$ Kupffer cells, motility parameters of T cells in the liver were not altered by chlodronate depletion of such cells. We observed two distinct CD 11 e+ populations within the liver parenchyma, one immotile surrounding portai vessels but in no contact with the lumen of such vessels, and another one present in the turnar sinusoids independently of their metabolic zonation and effectively patrolling the sinusoids.

Conclusions

Several peculiar patrolling behaviors of both T cells and dendritic cells (CDI $1e^+$ cells) within the livers in mice bearing subcutaneous tumors were discovered. These results set up a baseline to study such behaviors in mice undergoing immunotherapy treatments such as anti-PD-1, anti-CTLA-4 or anti-CD 137 and the preexisting response upon turnar celi arrival.

P68

Effect of isoflurane or ketamine-xylazine anesthesia on the dynamics of the murine immune response to influenza vaccination

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Background

Ketamine/xylazine (KX) and isoflurane are among the most used anesthetics in mouse research. Some evidence indicates that these drugs can alter the functionality of the immune system. However, little information is available about the effect of their prolonged and repeated administration on mouse vital parameters and on the immune response.

Methods

To clarify these aspects, we measured vital parameters and assessed immune functionality in the popliteal lymph node (pLN) of mice vaccinated against influenza virus and anesthetized for two hours with isoflurane or KX.

Results

We initially evaluated the minimal KX dose for surgical tolerance. Our results showed that KX anesthetized mice presented under-physiological levels of oxygen saturation (SpO2) and did not survive anesthesia. Importantly, administration of external oxygen restored SpO2 and reduced mortality. Moreover, we characterized the motility patterns of immune cells in the pLN using 2-photon intravital microscopy, a powerful technique that allows in vivo imaging at cellular level in organisms. Following this approach, we observed significant differences in the directionality and speed of neutrophils, T cells and B cells, in association with the anesthetic protocol employed. Interestingly, these observations correlated with the different levels of the inflammatory cytokines Interferon-γ and Interleukin-6 induced by KX and isoflurane. Additionally, the number of dying cells in the pLN, measured by flow cytometric analysis, significantly increased in all the anesthetized mice compared to the control group.

Conclusions

In conclusion, we found that isoflurane shows a reduced effect on the vital parameters, and it is more suitable for immune studies compared to KX.

P69

Transformer-based spatial-temporal detection of apoptotic cell death in live-cell imaging

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Background

Intravital microscopy has revolutionized live cell imaging by allowing the study of spatial-temporal cell dynamics in living animals. However, the complexity of the data generated by this technology has limited the development of effective computational tools to identify and quantify cell processes. Amongst them, apoptosis is a crucial form of regulated cell death involved in tissue homeostasis and host defense. Live-cell imaging enabled the study of apoptosis at the cellular level, enhancing our understanding of its spatial-temporal regulation. However, at present, no computational method can deliver label-free detection of apoptosis in microscopy time-lapses.

Methods

To overcome this limitation, we developed ADeS, a deep learning-based apoptosis detection system that employs the principles of computer vision and activity recognition. ADeS relies on a Conv-Transformer architecture in which a convolutional module extracts the features of the apoptotic cells, and an attention-based module evaluates the relationship between consecutive frames, allowing for effective classification of apoptotic sequences. We trained ADeS on extensive datasets containing more than 10,000 apoptotic instances collected both in vitro and in vivo.

Results

ADeS achieved a classification accuracy above 98% and outperformed state-of-the-art solutions for the quantification of cell death in live-cell imaging. ADeS is the first method capable of detecting the location and duration of multiple apoptotic events in full microscopy time-lapses, surpassing human performance in the same task. We demonstrated the effectiveness and robustness of ADeS across various imaging modalities, cell types, and staining techniques. Finally, we employed ADeS to quantify cell survival in vitro and tissue damage in vivo, demonstrating its potential application in toxicity assays, treatment evaluation, and inflammatory dynamics.

Conclusions

In conclusion, ADeS constitutes a novel solution for apoptosis detection that combines state-of-the-art microscopy and DL. Our findings suggest that ADeS is a valuable tool for the accurate detection and quantification of apoptosis in live-cell imaging and, in particular, intravital microscopy data, providing insights into the complex spatial-temporal regulation of this process. The successful implementation of ADeS represents a step towards the general application of AR methods to live-cell imaging, bridging two distinct fields and importing the advantages of automated routines to live-cell imaging. Further work could extend the proposed pipeline to other cell populations, types of cell death, and, potentially, cell actions.

P70

Anti-Pd-1 promotes Cd8 T cell responses in tumor-draining lymph nodes by inducing follicular helper T cell-dependent Il-4 release

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Background

While anti-PD-1 therapy targets intra-tumoral $CD8^+$ T cells to promote clinical responses in cancer patients, recent evidence suggests an additional activity in the periphery, in particular, in draining lymph node. However, the mechanism by which anti-PD-1 mAb act in lymphoid organs is unclear.

Methods

We use two different OVA-expressing tumor models in immuno-competent mice to decipher the mechanisms by which anti-PD-1 mAb acts in tumor draining lymph nodes.

Results

Here, we show that anti-PD-1 mAb enhances $CD8^+$ T cell responses in tumor-draining lymph nodes by stimulating cytokine production in follicular helper T cells (Tfh). In the two different OVA-expressing tumor models, we observed that anti-PD-1 increased the proliferation and effector functions of tumor-specific $CD8^+$ T cells in the draining lymph node. Using two-photon imaging of intact lymph nodes, we visualized that tumor-specific $CD8^+$ T cells contacting dendritic cells acquired a blastic phenotype in anti-PD-1 treated mice. Surprisingly, anti-PD-1 did not primarily target $CD8^+$ T cells but instead specifically bind Tfh cells in vivo, which are cells expressing the highest levels of PD-1 in lymph nodes. Anti-PD-1 acts on Tfh cells inducing their proliferation and their relocation outside germinal center; visualizing by lymph node immunofluorescence. Most importantly, anti-PD-1 mAb induces Tfh-dependent interleukin 4 production. Blocking IL-4 or inhibiting the transcription factor Bcl6 abrogated anti-PD-1 activity lymph nodes while injection of IL-4 complex was sufficient to recapitulate the activity of anti-PD-1. Finally, a similar mechanism was observed in a vaccine setting.

Conclusions

We propose that Tfh cells and associated cytokines, in particular interleukin 4, play a key role in the peripheral activity of anti-PD-1 mAb.

P71

Nanoscopic imaging of microglia phagocytosis by individual synaptic states

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Background

Microglia are the only resident immune cells in the CNS. These cells modify brain function and drive brain diseases through their ability to shift between highly phagocytic and less phagocytic states depending on the signals they receive from individual synapses. However, precisely how and why individual synapses influence local microglia activity remains a mystery.

Methods

Here, we leverage the experimental advantages of the visual system and employ brain cell-type specific genetic models to remove ‘don’t eat me cues’ from synapse of known types and assess microglia phagocytic outcomes. In parallel, we leverage a nanoscopic imaging technique we developed based on STORM imaging that allows for RApid Imaging of tissues at the Nanoscale (RAIN-STORM, Alabect et al., Cell Reports Methods, 2022). This approach allows us to assess the level of pro and anti-engulfment cues on single synapses.

Results

We have shown that neurons regulate microglia phagocytosis via a neuron-derived receptor-ligand pair–signal regulatory protein alpha (SIRP alpha) and CD47. We further show that neuronal diversity locally controls phagocytic variations of microglia in the brain. In particular, we show that presynaptic neuron subtype variations in neuronal SIRP alpha drive local microglia phagocytosis state in distinct retinorecipient regions of the brain.

Conclusions

This study is significant because it leverages single synapse imaging to uncover the fundamental role of neuronal diversity in microglia phagocytosis and establishes neuronal SIRP alpha as a targetable molecular cue to modulate microglial function in neurodegenerative diseases.

P72

Cecelia: An image analysis toolbox to uncover immune cell interactions and behaviour

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Background

Cell interactions are important for effective immune responses. Mapping these interactions in time and space is technically challenging and can be labour intensive. Here we present a multifunctional toolbox, termed Cecelia (**C**ell-**cell** **i**nteraction **a**nalyser), that allowed us to dissect spatiotemporal immune responses. We use this tool to dissect interactions between $CD8^+$ T cells and dendritic cells (DCs) in the context of HSV and LCMV infection.

Methods

We implemented the toolbox as a combination of R/shiny and python/napari that enables researchers to readily identify cell populations, behaviour and interactions in 2D or 3D static images and 3D timelapse images. To investigate the role of $XCR1^+$ DCs during early T cell priming, we utilised the XCR1-DTR system to specifically deplete this DC subtype and applied a combination of flow cytometry as well as confocal and intravital two-photon microscopy during HSV and LCMV infection. We primarily utilise the generalist deep learning package Cellpose for cell segmentation and the HDF5-based file format Anndata to store and further process object information with, among others, Hidden Markov Models, Leiden clustering, flow cytometry gating and neighbour detection.

Results

$XCR1^+$ DCs have been shown in several infection and tumour models to be instrumental for T cell priming. Using Cecelia to apply deep learning and advanced statistical approaches, we show that T cell priming is reduced in the absence of $XCR1^+$ DCs; yet, T cells were able to gather activation signals from other cells that resulted in defined areas of activation within the draining lymph node in the context of HSV infection. We further validated the broad utility of Cecelia for image analysis by identifying cell populations, interactions and stromal compartments from commonly used multiplex imaging techniques such as spectral unmixing, OPAL, IBEX and imaging mass cytometry.

Conclusions

We integrated the combination of different segmentation workflows to capture a variety of cell shapes, sizes and fluorescent signals within various imaging modalities. Cecelia is an open-source package that simplifies image analysis workflows that currently require complex or expensive combinations of software packages.

P73

Dock8 regulates cellular actin distribution to maintain cohesion during migration through confined environments

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Background

The ability to migrate through diverse tissues is a hallmark of T cells that is key to their function in surveillance and pathogen clearance. When navigating through 3D tissue, T-cells utilize an amoeboid migration mode, characterized by frequent cellular shape changes, low adhesion to the extracellular matrix, and high cell velocity. We previously showed that T cells isolated from children with loss-of-function mutations in dedicator of cytokinesis 8 (DOCK8) who present with primary immunodeficiency, die when migrating through dense 3D tissue microenvironments. Here we aimed to define cytoskeletal processes in T cells that enable the rapid adaptability of cell shape, while maintaining cell cohesion during migration through confined environments.

Methods

We studied the migration dynamics of primary Dock8-/- and wild type (WT) T cells isolated from mice expressing both a nucleus reporter (nTnG) and F-actin reporter (lifeAct). Using 1D, 2D, and 3D *in vitro* assays paired with live cell microscopy, we examined nuclear and cell morphology, cell speed, and changes in the cytoskeleton under confinement.

Results

Dock8-/- T cells become entangled, have elongated nuclei, lose cell cohesion, and shatter when migrating through dense 3D tissue microenvironments, such as the skin, but migration in 2D in the absence of confinement is entirely normal. In fact, we found that in obstacle-free environments, Dock8-/- T cells actually migrate at even faster speeds than their WT counterparts. We showed that prior to entanglement, Dock8-/- T cells have normal organization of organelles and microtubule structures, but displayed a striking perturbation in the distribution of polymerizing F-actin within the cell compared to WT T cells. We identified a perinuclear pool of F-actin in WT T cells that is mechanoresponsive – emerging only in a confinement-dependent manner. In contrast, in Dock8-/- T cells we made the surprising observation that this central F-actin pool is entirely absent and instead, F-actin accumulates primarily at the cell's leading edge.

Conclusions

In summary, our data show that DOCK8 is a critical regulator of T-cell cohesion and survival during migration through complex environments by regulating the intracellular F-actin distribution. We identify a novel mechanism by which Tcells integrate mechanical information from their environment, distributing F-actin pools accordingly, and without which T-cells are unable to successfully navigate dense tissues.

P74

Rearrangement of the Mitochondrial Calcium Uniporter Complex is Essential for T-Cell Mediated Immunity

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Background

A fundamental process involved in the regulation of important T-cell functions includes a balanced cytosolic Ca^{2+} increase occurring upon TCR activation. Mitochondria play a crucial role in this context and support the elevated bioenergetic demands by taking up Ca^{2+}. Therefore, alterations in the protein machinery which regulates mitochondrial Ca^{2+} ($_{m}Ca^{2+}$) influx across the inner mitochondrial membrane, i.e. the mitochondrial calcium uniporter (MCU) complex, can be implicated in regulation of T-cell immunity. However, the role of MCU in T-cells is not entirely apprehended.

Methods

Primary naive CD4 T-cells were isolated from healthy human donors and activated for 72 hours. Gene and protein expression techniques were used to determine the molecular composition of MCU in naive and effector T-cells. Single-cell live fluorescence microscopy was utilised to evaluate $_{m}Ca^{2+}$ in cells overexpressing MT3.1 Pericam, ROS in cells overexpressing HyPer, mitochondrial membrane potential using TMRE, and ATP using MitoATP-red. Mitochondrial respiration was determined by Seahorse assays. Transcriptome and proteome screening revealed metabolic changes occurring after cell activation. MCUa knockdown effector T-cells were utilised to examine the role of MCU in cell metabolism and function. Cytokine expression and release were evaluated, along with metabolic and functional parameters. Finally, primary rat MCUa knockdown CD4 T-cell lines overexpressing Scarlet-GCaMP6S were generated using CRISPR/Cas9. An *in vivo* model of multiple sclerosis (experimental autoimmune encephalomyelitis-EAE) was used to determine the role of MCU in autoimmunity.

Results

Our results show that the MCU complex is rearranged upon activation of CD4 T-cells; an event evoking elevated $_{m}Ca^{2+}$ uptake and mitochondrial bioenergetic output. Transcriptome and proteome analyses of naive and effector cells reveal an essential role of mitochondrial metabolism during T-cell activation, in a time dependent manner. MCUa knockdown diminishes $_{m}Ca^{2+}$ and suppresses respiration, membrane potential, ATP production, IFNγ release and invasion in vitro. Additionally, mitochondrial protease AFG3L2 is upregulated in $MCUa_{KD}$ cells, through a mechanism involving EMRE. *In vivo*, MCUa downregulation in rat T-cells causes delayed EAE onset and a reduction of disease severity.

Conclusions

In summary, $_{m}Ca^{2+}$ influx through MCU is essential for proper T-cell function and is involved in autoimmunity.

P75

CD4+ T cells show a cytolytic phenotype functions in human visceral leishmaniasis

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Background

Control of Visceral leishmaniasis (VL), a parasitic disease caused by *L. donovani*, requires robust $CD4^+$ T cells response to control the parasite replication by IFN-γ production and activation of macrophages. However, in VL patients, the anti-parasitic $CD4^+$ T cell responses are ineffective because of unknown reasons. Our recent study on transcriptional signature of $CD4^+$ T cell isolated from peripheral blood of active VL patients showed enhanced expression of genes related to cytotoxicity. In present study, we investigated expression of these cytotoxic molecules including granzyme B (GZMB), Granulysin, Perforin and NKG7 on different $CD4^+$ T cell subsets and established the role of GZMB in regulating $CD4^+$ T cells functional capacity.

Methods

Peripheral Blood mononuclear cells were isolated from active and post treated VL patients and endemic controls and flow cytometry was performed. LAMP assay (CD107b expression) was performed to study the degranulation capacity of $CD4^+$ T cells. Whole blood assay was performed to study the antigen specific cytokine production by $CD4^+$ T cells and their association with cytotoxic molecules. Enzyme linked immunosorbent assay (ELISA) was performed to measure circulatory as well as antigen specific GZMB production. Concanamycin A (Con-A), a known v-ATPase inhibitor was used to examine the effect of GZMB inhibition on $CD4^+$ T cells function.

Results

We found that activated and degranulating $CD4^+$ T cells ($CD38^+CD107^+$) had higher expression of GZMB, Granulysin, perforin and NKG-7. Similarly, GZMB level in plasma and antigen stimulated whole blood culture supernatant were significantly elevated in active VL patient compared to post treatment and EC. There was no change is IFN-γ secretion when whole blood culture was stimulated with soluble leishmania antigen in presence of Con-A but a significant decline in IL-6 production was observed.

Conclusions

VL $CD4^+$ T cells show a cytolytic phenotype and further investigations are required to understand their differentiation and function, particularly for promoting anti- parasitic immunity for host protection and effective intervention or therapy.

P76

Mast cell tissue seeding and homeostasis critically depends on mitochondrial fusion

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Background

Mitochondrial fission and fusion dynamics are key regulators of cellular homeostasis, differentiation and activation by shaping the metabolic and biochemical landscape of cells. In recent years, a renaissance of the role of metabolism in immune cells occurred with a strong focus on mitochondrial biochemistry and dynamics. However, for mast cells (MCs), long-lived master regulators of anaphylaxis and contributors to other immune responses, the functional roles of metabolic and underlying cell organellar changes for their population development in vivo have remained unknown.

Methods

As mitochondrial morphological states of immune cells have never been directly examined in living tissues, we generated reporter mice to visualize mitochondria in tissue-resident MCs and macrophages. We used two-photon intravital microscopy to analyze mitochondrial structure and dynamics of MCs and macrophages in the mouse ear dermis. Furthermore, we generated conditional knockout mouse models to specifically deplete key regulators of mitochondrial fission (Drp1) and fusion (Mfn1/Mfn2, Opa1) in MCs. To address the mechanistic aspects of our observed in vivo phenotypes, we cultured peritoneal MCs for the use with high-resolution imaging, live cell imaging (e.g. photoconversion), (sc)RNAseq analysis and various metabolomics measurements.

Results

In contrast to tissue-resident macrophages, endogenous MCs show a tubular mitochondrial network in vivo. When interfering with this fused mitochondrial state in genetic mouse models, we make the surprising finding that MCs disappear almost completely from the connective-tissue niches (dermis, peritoneum, tongue) of adult mice. However, MC populations in neonate mice remain unaffected. Our in vitro experiments with gene-deficient MCs or chemical inhibition reveal a critical role of OPA1 in cell cycle arrest, cell death induction and MC-specific marker expression, e.g. key transcriptions factors GATA2 and MiTF. Surprisingly, the metabolic changes in OPA1-deficient MCs were only mild. Instead, we find that the loss of OPA1 associates with a fatal induction of the integrated stress response (ISR), which appears to drive MCs into cell cycle arrest and cell death, and can be rescued by the ISR inhibitor ISRIB.

Conclusions

Mitochondrial fusion is a key process for MC homeostasis and population development, comparable in its importance to the well-known GATA2-driven transcriptional program and Kit ligand/KIT-mediated survival signaling.

P77

HIV Nef undermines CD4 T helper functions towards B cell and CTL responses

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Human immunodeficiency virus (HIV)-infected patients fail to produce broadly neutralizing antibodies and develop suboptimal, non-sterilizing cytotoxic T lymphocyte (CTL) responses and thus display pronounced defects in their adaptive immune response. As both, B cell and CTL responses depend on functional CD4 T cell help and CD4 T cells are the main HIV target cells, we assessed how HIV infection affects their helper functions. We focused on the HIV pathogenicity factor Nef as prime candidate for a viral effector that impairs immune responses, since Nef is known to interfere with cardinal CD4 T cell functions such as cell motility, immunological synapse organization, T cell receptor signal transduction and intracellular protein trafficking. To test this hypothesis, we expressed Nef in antigen specific primary murine CD4 T cells by ex vivo MLV transduction. Subsequent adoptive cell transfer to transgenic mice allowed us to assess the role of Nef in different steps of the adaptive immune response, from CD4-dendritic cell (DC) interactions to downstream B cell or CD8 T cell responses.

Assessing the effect of Nef on interactions of CD4 T cells with cognate peptide-loaded DCs by intravital imaging and in vivo proliferation analyses, revealed slower migration but longer interactions of Nef-expressing cells, which was associated with mildly reduced cell activation at unaltered proliferative capacity. Using immunization approaches to assess downstream effects of Nef on B cell responses, we found intact follicular helper cell differentiation, but strongly impaired interactions with cognate B cells in vitro and in vivo, failure in germinal center induction, and lack of antibody production. Nef-dependent impairment of antibody production to recall antigen in HIV-infected human tonsil histocultures confirmed Nef as key determinant of HIV-1 induced disruption of CD4 T cell help to B cells. Using lymphocytic choriomeningitis virus infection as a model to assess the impact of Nef on CD4 T cell help for functional CTL responses, we found CTL generation, viral clearance, functional differentiation and cytotoxicity to be reduced by Nef. These effects were significant during acute LCMV infection but even more pronounced during viral rechallenge.

Collectively these results demonstrate that HIV infection impairs adaptive immune responses by limiting helper functions of infected CD4 T cells and suggest HIV pathogenicity factor Nef as main driver of this immune evasion mechanism.

P78

Organization and function of DC centrosomes during immune synapse formation

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Background

Centrosomes are microtubule-nucleating organelles consisting of twO centrioles which are surrounded by pericentriolar material. In interphase the number of centrosomes is strongly regulated to one centrosome whereas the centrosome duplicates before a cell enters mitosis in order to establish the two spindle poles and partition the genetic material. The appearance of multiple centrosomes was reported in lipopolysaccharide- (LPS-) matured antigen-presenting dendritic cells (DCs) despite these cels are cell cycle arrested (Weier et al., JCB, 2022). Recently, members of my group analysed DC effector functions and published that DCs with multiple centrosomes show enhanced T cell activation and enhanced persistent locomotion in comparison to DCs with one centrosome (Weier et al., JCB, 2022).

Methods and Results

My PhD project focuses on analysing the role of multiple centrosomes for T cell activation as well as the underlying molecular mechanisms. Therefore, I quantify T cell activation on a cell population level and on a single cell level in dependence of DC centrosome numbers by measuring T cell proliferation and activation marker upregulation. Using distinct methodological set-ups we show that DCs with multiple centrosomes harbor an enhanced T cell activation capacity in comparison to DCs with only one centrosome. This result is further underlined by a mathematical model for T cell priming. In a second part, I want to understand the organization of the DC centrosome during inmmune synapse formation. I use live cell imaging to visualize the configuration and spatio-temporal dynamics of centrosomes upon antigen-specific T cell contact. With this I was able to show on a single cell level that multiple centrosomes cluster during antigen-specific T cell activation. Moreover, in contrast to the T cell's centrosome, the DC centrosome does not re-orient towards the T cell contact side - regardless of whether the DC has one or multiple centrosomes.

Conclusions

In further experiments, the microtubules which are emanating from the centrosome will be studied in the context of immune synapse formation. To analyse the role of the centrosome during immune synapse formation, models for centrosome amplification but also perturbation of centrosome integrity will be established.

P79

Predicting CD8+ T cell clonal dynamics in the tumour immune response

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Background

In the face of chronic immune challenges, such as cancer, activated CD8+ T cells infiltrate the target tissue, proliferate, and clonally expand. However, the dynamics of these clonal populations - whether they continue to expand, or contract and disappear, or retain memory cells, is unknown.

Methods

Here, we employ a bilateral tumour mouse model to track the dynamics of endogenous CD8+ T cells sustaining the tumour immune response. By sampling the same clonal populations, on different days, in the same individual, we identify expanding and contracting clonal populations.

Results

Through analysis of these populations, we demonstrate that single time point gene expression data can accurately predict the future expansion rate of any clonal population. We further show that contracting clonal populations retain a pool of revivable memory-like cells in the tumour.

Conclusions

As well as identifying a novel immunotherapeutic target, our results facilitate the development of new methods to faithfully reconstruct immune activity from single time point data.

P80

Dynamics of initial immune responses to pioneering metastatic tumor cells in the liver tissue

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Background

Tumor metastases are the main cause behind cancer related death. Liver is one of the most frequently targeted organs in cancer metastases. The presence of liver metastases has been shown to promote systemic immunosuppression and poor response to immune checkpoint blockade immunotherapy. We set up intravital imaging approaches to visualize and characterize the types of immune responses that metastatic tumor cells drive when arriving in the liver tissue in order to understand how spatiotemporal dynamics of liver specific immune responses dictate the outcome of immune responses to established metastases.

Methods

Performing intravital microscopy in several immune-cell reporter mice we characterized the dynamics of immune responses against tumor cells from syngeneic colorectal cancer and melanoma cell lines after their intrasplenic injection. NK, T cell, dendritic cell, tumor and Kupffer cells behavior was tracked by time lapse microscopy videos and immunofluorescence.

Results

Tumor cells arriving to the liver rapidly give rise to vesicles that are uptaken by circulating or liver resident leukocytes. Kupffer cells frequently perform efferocytosis of large tumor cell pieces and were the main cells keeping fluorescent antigen in the liver 24 hours after tumor cell arrival. Few cells interact with arriving tumor cells and no active migration towards metastatic tumor cells or clusters was observed from NK cells or T cells. Progressive enrichment on T cells but no NK cell performing intravascular patrolling was observed following 24 hours after tumor cell arrival. Such T cells form intravascular clusters in areas where tumor cell antigenic material is observed, mainly uptaken by F4/80 positive cells, and not around clusters of tumor cells populating the liver vessels. The formation of such intravascular clusters could be abolished by pertussis treatment or chlodronate treatment. Circulating F4/80 macrophages rapidly repopulated the livers and were observed in intravascular clusters with T cells 48 hours upon tumor cell arrival. Importantly, antigen specific OTI tumor cells were driven towards these intravascular clusters independently of their antigen recognition.

Conclusions

We have visualized for the first time the dynamics of immune responses against tumor cells arriving to the liver and showed tissue specific immune responses as intravascular T cell clusters that may dictate the final outcome of metastatic cells in the liver.

P81

Dynamic micropatterns as a novel tool to study immune synapse formation and early BCR signalling

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Background

B lymphocytes form a critical part of the adaptive immune system. Their activation and differentiation into antibody-producing cells is dependent on the B cell receptor (BCR), which recognizes a vast repertoire of foreign antigens. V/hile B cells can be activated by soluble antigens, it has emerged that probably the most t¡pical form of antigen encounter is on the surface of antigen presenting cells (APC), which leads to the formation of the immune synapse (IS).

Methods

To study the formation of the IS and the transmission of BCR signalling we use a novel dynamic biotinylation-based micropatterning technique. This method allows us to observe and image individual B cells before and after BCR engagement with high spatio-temporal control of the process. We can analyse both activatory (surrogate antigen) and non-activatory areas in the cell as well as different time points of the activation process. We can also introduce other factors like mechanical stress in the activation process by using different micropaffern shapes. This method allows for different microscopy modalities both in fixed and live samples.

Results

Using the dynamic micropatterns, we can eflìciently visualize differential organization ofvarious activated BCR signaling molecules on the IS interface separated in non-activatory and activatory areas. For example, the activated signaling subunit of the BCR, phosphorylated CD79A, robustly changes its localization from the non-activated to the activated area upon dynamic administration of surrogate antigen and the following cell spreading. Live imaging of the B cell actin cytoskeleton upon IS formation shows highly effîcient cell spreading with the use of filopodial protrusions as seeds of leading edge.

Conclusions

Mimicking the formation of the IS, the dynamic micropatterns allow visualization of B cell activation in a highly synchronous manner. The system grants high-resolution imaging and detailed monitoring of the activation of these very small cells. The technique allows the analysis of the intensities and spatial distributions of different critical signalling proteins during the early cell activation process. Moreover, the possibility of using micropatterns with different shapes and sizes can be used to induce polarization or mechanical stress and study the influence of these factors in B cell activation.

P82

Flt3L availability in local niches regulates spatiotemporal organization and development of resident dendritic cell networks

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Background

Conventional dendritic cells (cDC) are essential players for the initiation of adaptive T cell responses in lymph nodes (LNs). Distinct subsets of cDC form dense networks in LNs but the spatiotemporal organization and development of cDC networks are poorly characterized.

Methods

Focusing on XCR1+ type 1 cDC (cDC1), we used genetic lineage tracing, specific depletion, photoconversion, in vivo antibody labelling and single cell RNA sequencing to investigate the generation and maintenance of cDC networks. In addition, we generated a novel mouse model to pulse label a wave of cDC precursors (preDC) in the bone marrow, which allows the analysis of spatiotemporal cDC development.

Results

Here, we show that preDC predominantly home to LN medulla, in contrast to T cells that home to LN paracortex. In the medulla, preDC developed into fully functional immature cDC1 over several days and gradually migrated from medulla to paracortex as they develop. During inflammation, majority of the immature cDC1 matured and migrated towards the paracortex. This synchronized response reduced the number of fully functional immature cDC in the medulla, which reduced the antigen uptake capacity of the LN. Interestingly, reduced immature cDC1 numbers after inflammation or cDC1 depletion in turn increased the local availability of Flt3L, a key cytokine for cDC development, specifically in the medulla as the number of consumers for this cytokine decreased in this niche. Finally, increased Flt3L signaling accelerated the local preDC-to-cDC1 development to rapidly reestablish the disrupted cDC network.

Conclusions

In summary, our findings reveal key principles of the spatiotemporal development of cDC in LNs and describe a local feedback mechanism for the maintenance of cDC networks, which may guide novel angles for immunotherapy.

P83

Mechanisms of lymphatic vessel branching morphogenesis

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Background

Branching morphogenesis is a ubiquitous process that gives rise to high exchange surfaces in the vasculature and epithelial organs. Lymphatic capillaries form branched networks, which play a key role in the circulation of tissue fluid and immune cells. Although mouse models and correlative patient data indicate that the lymphatic capillary density directly correlates with functional output, i.e. tissue fluid drainage, adaptive immune response, and resolution of inflammation, the mechanisms ensuring efficient tissue coverage remain poorly understood.

Methods

Here, we use the mouse ear pinna dermis as a model tissue and combine lineage-tracing, genetic perturbations, whole-organ reconstructions and theoretical modeling to study the structure of lymphatic capillary network and the mechanisms that assure efficient space-filling by the growing lymphatic capillaries.

Results

Our results show that the dermal lymphatic capillaries tile space in an optimal, space-filling manner. This coverage is achieved by two complementary mechanisms: initial build-up of a non-optimal global scaffold, followed by rapid optimization of local coverage. With these two ingredients, we show that a minimal biophysical model can reproduce quantitatively whole-network reconstructions, across development and perturbations. Detailed results will be presented in the Workshop.

Conclusions

Our results show that lymphatic capillary networks can exploit local mechanisms to achieve tissue-scale optimization.

P84

Dynamic characterization of biodistribution of novel immunotherapeutics using 2-photon intravital microscopy

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Background

Biodistribution studies investigate the selective dissemination of a molecule in an organism in time. To reach this goal, imaging methods are commonly used, including MRI, PET, or CT scan. These technologies provide overall pictures of the entire organism, or parts of it, from a macroscopic point of view, allowing to assess the overall spread of a drug, its organ specificity, and possible off-target distribution. However, macro-imaging methods usually present poor sensitivity, low-resolution images, and limited capacity to evaluate intra-organ and cellular distribution. On the contrary, micro-imaging techniques, despite lacking a multi-organ perspective, are commonly applied to study cellular interactions within tissues. Such information are often neglected in biodistribution studies, but are essential to understand the mechanisms of action of novel therapies. Among the microscopic techniques, 2-photon microscopy (2PM) presents several advantages compared to other methods, such as high tissue penetration, low photo-damage, and the introduction of the time factor to images otherwise static, thanks to intravital microscopy (2P-IVM). One of the applications of 2P-IVM is to observe the arrival of drugs to the target organs in real time. Importantly, drug delivery time is essential in biodistribution studies to optimize administration protocols. However, the use of 2P-IVM to analyze drugs biodistribution has been so far limited.

Methods

In this work, we applied a mixed 2P/confocal microscopy based approach to study the biodistribution of different immune-targeting drugs, including nanoparticles and therapeutic antibodies, in mouse models of vaccination and cancer.

Results

First, we observed and quantified the precise time of arrival of each drug to the target organ, the lymph node (LN). Next, we analyzed the suborgan preferential localization of each molecule in the LN areas, highlighting drug specific distribution patterns. Moreover, we evaluated with cellular detail the interaction of the drugs with different immune cells and LN vasculature. These distribution features were strictly linked to each drug target. Finally, we used 2P-IVM to analyze the dynamic behaviour of the target cells and confirm drug immune-activation functions.

Conclusions

With these results we showed how 2P-IVM can be applied to study in vivo the biodistribution and the effect of novel therapeutics from organ to cellular level.

P85

DiLiCre, a double locked light-inducible Cre recombinase for real-time imaging and positional-cell tracing in vivo

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Background

The experimental need to engineer the genome both in time and space, has led to the development of several photoactivatable Cre recombinase (paCre) systems. Although paCre systems have been successfully applied to examine cells and tissues derived from paCre mouse models, the use of a transgenic mouse line for e.g., positional-cell tracing of cancer and immune cells requires further improvements of the optimal tradeoff between photo-induced and background activity of the Cre recombinases. Here, we engineered an optimized photoactivatable Cre recombinase system that we refer to as doxycycline- and light-inducible Cre recombinase (DiLiCre) to develop an efficient paCre recombinase mouse model..

Methods

DiLiCre1.0 was adapted and engineered from a construct recently published by Zhang et al. (2017, PhoCl). The signal-to-noise was optimized using FLEx reporters, flow cytometry, and live confocal imaging. To further control the inefficient and non-intentional background recombination, we engineered an upgraded version referred as DiLiCre2.0, by introducing a tandem ERT2 repeat. C57BL/6J embryonic stem cells (mESC) were targeted at *Col1a1* locus to generated the DiLiCre2.0 mouse line. Flow cytometry and live imaging were performed in mESC and DiLiCre2.0;R26-Confetti derived intestinal organoids to conduct the ex vivo validation. Photoconversion of intestinal crypts and lineage tracing experiments of skin cells were performed on a Leica SP8 Dive system equipped with four tunable hybrid detectors.

Results

The double locked light-inducible Cre recombinase DiLiCre1.0 allows the control of the Cre activity in time and space. The addition of a second ERT2 repeat contributes to improve the signal-to-noise ratio by reducing the background recombination whilst maintaining the efficiency of light induction. Importantly, DiLiCre2.0 intestinal organoids recombine ex vivo with high efficiency and DiLiCre2.0;R26-Confetti mouse model can be used to perform positional-cell tracing in the intestine *in vivo*. DiLiCre2.0 mouse model is an efficient optogenetic tool to induce oncogenic cell transformations *in vivo* and trace the fate of fluorescently labelled cells upon DiLiCre2.0 activation.

Conclusions

Our data illustrates that the characteristics of DiLiCre2.0 are optimal for *in vivo* experiments and illustrated the biological applicability of DiLiCre for light-induced genetic editing *in vivo* and positional cell-tracing by intravital microscopy.

P86

Chimeric antigen receptor oligomerization

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Background

Chimeric antigen receptor (CAR) T-cell therapy is a successful immunotherapy for hematologic malignancies, with some limitations in terms of efficacy and reduction of relapse in certain cancers. Not to mention, the need to develop new CAR-T cell therapies that would target solid tumors and alternative antigens. Our goal is to achieve more potent CARs by taping into the not yet well understood strategy of receptor clustering promotion. This has already been demonstrated by Fleishman et al. by using programmed membrane proteins that would form dimeric, trimeric, and tetrameric complexes to promote bigger receptor clusters and more stable and effective immunological synapse formation and signaling.

Methods

All our constructs had been expressed in primary human T cells. We have successfully performed killing assays targeting hematological malignancies using Raji and K562 cells modified to express CD19 antigen as a model cell line. We are currently working on establishing solid tumor 2D and 3D models with MCF-7 and SH-SY5Y cells, where killing dynamics and T-cell infiltration will be determined using the CellInsight™ CX7 High Content Analysis Platform. The formation of immunological synapses between different constructs was observed using Leica TCS SP5 Confocal Laser Scanning Microscope, by applying fluorescent proteins and antibodies.

Results

We engineered stronger CARs by exchanging the clinically used CD8 transmembrane domain with novel domains promoting diverse oligomerization orders between receptors. The second strategy of elevating oligomerization was to add different oligomerization-encouraging proteins to the c-terminal end of the CAR construct. We have demonstrated the efficacy of our contract with various antigen targets such as Cluster of Differentiation 19 (CD19), human epidermal growth factor receptor 2 (HER2), and Disialoganglioside (GD2). Our oligomeric-modified CARs outperformed the second generation CAR using 4-1BB and CD3z intracellular domains (an example of this is FDA approved Kymriah CAR-T, with an scFv against CD19) cell therapy in the in vitro killing assays.

Conclusions

Proving that the promotion of CAR oligomerization and clustering does translate into a stronger cytotoxic activity. The divergence in oligomerization is also evident in the immunological synapse formation.

P87

Cryo-Expansion Microscopy to Unveil the Molecular Architecture of the Immune Synapse

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Background

Cytotoxic T cells destroy cancer cells by creating a nanoscale gap between them calied the immunologica! synapse (IS). At this specialized junction, the correct organization of molecules in distinct zones is crucial for optimal T celi function. Achieving this requires a large 3D rearrangement of the T cell's actin and microtubule cytoskeleton, which also polarizes the centrosome, consisting of two centrioles, as weli as lytic granules to the IS. Super-resolution fluorescence microscopy has revolutionized our understanding of the IS, but it is not routine due to the high cost of equipment and expertise required. Thus, we undertook to set-up an easy-to-implement super-resolution microscopy method for IS imaging.

Methods

We recently developed Cryo-Expansion microscopy (Cryo-ExM), a novel approach that preserves celiular ultrastructure and aliows for super-resolution imaging using conventional diffraction-limited microscopes. We used Cryo-ExM to image morphological changes in Jurkat and primary human T celis on activated surfaces and T-celltumor celi pairs.

Results

We demonstrated that these · immune celis are isotropically expanded. Cryo-ExM revealed specific molecular architecture rearrangements of the IS, including actin and centrosome polarization, as weli as its maturation status. Cryo-ExM also uncovered an unexpected modification of centriole architecture in the Jurkat immune celi model, with short centrioles lacking appendage proteins, which may prevent centriole docking to the IS membrane. Primary T cells displayed as well shorter centrioles but retained distai appendages and lacked sub-distai .appendages. Whether these modifications affect centrosome function in IS formation is under current investigation. Finally, we are mapping the IS molecular organization of CD45, CD2, pZAP70, Lck, and markers of the lytic granules.

Conclusions

Cryo-ExM provides a new approach for super-resolution imaging of the IS in Jurkat and primary human T cells, enabling molecular imaging of the IS on activated surfaces or in interaction with a turnar target celi. This powerful technique allows for the uncovering of structural and molecular details of immune cells, thus revealing novel insights into T cell biology. It has the potential to become a useful tool for preclinical characterization of engineered T celi products.

P88

Ligation-induced TIGIT nanoclusters coalesce with the TCR and signal via its ITT-like domain to inhibit T cells

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TIGIT is an inhibitory receptor expressed on lymphocytes and can inhibit T cells by preventing CD226 co-stimulation through interactions in cis or through competition of shared ligands (e.g., CD155). Whether TIGIT directly delivers cell-intrinsic inhibitory signals in T cells remains unclear. CyTOF analysis of lymphocytes from matched human tumour and peripheral blood samples revealed that TIGIT and CD226 co-expression is rare on tumour-infiltrating lymphocytes. Using confocal, FRAP, STED, TIRF and STORM microscopy, we demonstrate that ligation with CD155 causes TIGIT to reorganise into dense, dynamic nanoclusters, which coalesce with T cell receptor clusters at immune synapses. Functionally, this reduces activation-induced cytokine secretion in a manner dependent on TIGIT's intracellular ITT-like signalling motif. Though clustering of TIGIT was solely dependent on its ability to bind its ligand, signalling-deficient mutants of TIGIT surprisingly increased levels of activation when stimulated with CD155-expressing target cells. Thus, TIGIT can directly inhibit lymphocyte activation, acting independently of CD226, requiring intracellular signalling that is proximal to the TCR. In the context of tumours where infiltrating lymphocytes do not co-express TIGIT and CD226, TIGIT likely inhibits T cells predominantly through direct T-cell intrinsic inhibitory signalling and preventing costimulatory signals to CD226+ populations.

P89

Intravital Multiphoton Microscopy Reveals Immune Cell Interactions in Models of Cancer and Disease

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Intravital Multiphoton Microscopy allows for the direct visualization of single cell behavior in its true microenvironment. Since the development of the technology, many studies have concentrated on immune cell interactions in models for cancer and disease. Early studies were able to visualize macrophage's role in tumor cell extravasation in multiple models of breast cancer. Using other techniques, this interaction was shown to be a paracrine loop between the EGF and CSF-1 receptors. Other imaging experiments have shown macrophage involvement in fibrosis on macroparticles beneath the peritoneum and stromal cell interaction in pancreatic cancer increasing the oxidation state of the tumor cells, using NAD(P)H and FAD as a readout.

After using intravital imaging of the blood brain barrier to determine the best polymer based lipid nanoparticle, we are now studying how microglia are involved in transport of these nanoparticles across the blood brain barrier. And in the newest studies, after molecular biology technique showed that aneuploidy cells in the small intestines show increased antigen presentation, we are now attempting to visualize CD8+ T-cell interaction with aneuploidy cells in the intestines of live mice. By taking advantage of multiple methods of fluorescently labeling cells in live mice, from transgender models to antibodies, dextrans and nanoparticles, and the least invasive surgical procedure possible, most models of cancer and disease can be imaged.

P90

Macrophages in the synovial lining niche initiate neutrophil recruitment and articular inflammation

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Background

Rheumatoid arthritis (RA) is a chronic autoimmune disease of the joints, characterized by leukocyte recruitment into the synovium, the joint-supporting tissue, as well as the articular cavity (Smolen et al., 2018). Healthy synovium consists of the lining layer which surrounds the joint cavity and the sublining layer which is positioned deeper in the tissue. Both layers contain resident macrophages with substantially different phenotypes shaped by their microenvironment (Alivernini et al., 2020, Culemann et al., 2019, Zhang et al., 2019). However, it remains unclear how these distinct resident macrophages become activated in RA, i.e. what constitutes the break of tolerance in these cells leading to pathogenic leukocyte recruitment.

Methods

In this study we employ state of the art confocal microscopy and image analysis in a systemic, whole organ and quantitative way to dissect the first cellular and molecular events at the onset of joint inflammation. Further, we utilize FACS, RNA sequencing, and a unique mouse model with cell-specific deletion of a key transcription factor to present the evidence that synovial inflammation begins with the activation of lining macrophages.

Results

We show that lining but not sublining macrophages phagocytose immune complexes containing the model antigen. Using the antigen-induced arthritis (AIA) model, we demonstrate that on recognition of antigen-antibody complexes, lining macrophages undergo significant activation, which is dependent on Interferon Regulatory Factor 5 (IRF5), and produce chemokines, most notably CXCL1. Consequently, at the onset of inflammation neutrophils are preferentially recruited in the vicinity of antigen-laden macrophages in the synovial lining niche where vascular endothelium expressed the activation marker E-selectin. As inflammation progresses, neutrophils disperse across the whole synovium and form swarms in synovial sublining during resolution.

Conclusions

Our study highlights lining macrophages as critical upstream instigators of joint inflammation and warrants further investigation into how the cell-crosstalk in the lining can be targeted to abridge inflammation.

P91

Age-related structural and molecular defects in the thymic epithelium impair regeneration of the involuted thymus

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Background

Age-associated thymic involution and the resulting decline in T cell production impairs immune reconstitution in cancer patients and is linked to immunosenescence. Thymic epithelial cells (TECs) orchestrate thymic involution and pharmacological or genetic targeting of TEC growth pathways can promote thymic regeneration. However, none of these approaches provide for sustained regeneration of thymic function, highlighting a poor understanding of the mechanisms of age-associated thymic involution. This impasse is partly due to the lack of robust methods for resolving TEC within the context of the thymic microenvironment.

Methods

To study the TEC microenvironment, we generated novel fluorescent reporter mice and developed a pipeline to quantify TECs *in situ* using light-sheet and confocal microscopy. In conjunction, we used whole organ 3D imaging to study the structure and dynamics of the TEC microenvironment during involution and regeneration.

Results

We report that standard flow cytometric approaches greatly under-estimate the number of TECs. We also discovered an atypical TEC population emerges with age and expands with thymic involution. These age-associated TECs (aaTECs) were morphologically distinct from their cortical and medullary TEC counterparts, forming highly compact structures that exclude developing T cells, indicating that aaTECs contribute to age-associated thymic dysfunction. Moreover, the involuted thymus showed impaired thymic regeneration following acute injury, while the aaTEC population expanded in number and volume. These data suggest that this unique feature of thymic involution impairs immune reconstitution. Subsequent single-cell RNA-seq analyses identified a unique transcriptional profile in aaTEC, characterized by a partial epithelial-to-mesenchymal transition (pEMT).

Conclusions

We conclude that the aaTEC is a key feature of thymic involution that limits immune function and reconstitution.