Abstracts: selected short talks

Chronically painful versus regenerating nerve injury is associated with differential myeloid cell states

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Neuroimmune interactions play a role in numerous chronic pain conditions including neuropathic states. However, much is still unknown regarding the exact mechanisms underlying these communications and the cell types involved. We have recently demonstrated that myeloid cells remain persistently elevated at the site of injury following induction of chronic neuropathic pain, using the partial sciatic nerve ligation (PSNL) model (Liang, Hore et al. 2020). Here, we aimed to test whether particular myeloid populations are specific to neuropathic pain and are not simply associated with nerve injury in general. For this, we performed either PSNL or sciatic nerve crush, a model of regeneration which is not associated with chronic neuropathic pain, on C57BL/6J mice (n=2). Nerve tissue was harvested 1-week following surgery, and single cell RNA sequencing was conducted on FACS sorted CD45+/CD11b+/Ly6G- live cells using a 10X Chromium platform. Resulting reads were aligned with Cell Ranger, and data were further analysed using the R package Seurat. Though in-depth analyses are still underway, our results to date indicate substantial differences between the models. Namely, two cell clusters which upregulate pro-inflammatory genes including Spp1, S100a8/a9 and Il-1β, are expressed almost exclusively by PSNL nerve. Meanwhile, much larger clusters of cells which upregulate anti-inflammatory and pro-resolving genes, including Alox15 and Apoe, are present following crush. In conclusion, our data indicate that there are myeloid cell populations specifically associated with non-resolving, chronically painful nerve injury. Studying their phenotype in more detail may help identify key genes and pathways suitable for analgesic drug development.

Phenotype of dorsal root ganglia macrophages in a model of arthritis pain

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Background/aims: Pain is a persistent feature of rheumatoid arthritis (RA). We hypothesise that sensory neuron-macrophage crosstalk in the dorsal root ganglia (DRG) plays a fundamental role for maintenance of RA pain. In the K/BxN serum-transfer model of inflammatory arthritis, we observed that hind paw hypersensitivity (allodynia) persists after joint swelling is resolved. This phase is associated with presence of CX3CR1-macrophages into the DRG. Here, we assessed the development of K/BxN serum transfer allodynia in CX3CR1GFP/+ (WT) and CX3CR1GFP/GFP (KO) and phenotyped DRG macrophages.
Methods: Following K/BxN serum transfer, hind paw arthritic scores and mechanical thresholds (von Frey test) were assessed in WT and KO mice for up to 28 days. Cervical and lumbar DRG were obtained to isolate macrophages (CD45+ CD11b+F4/80+ cells) which were quantified and phenotyped by flow cytometry. Bone marrow-derived macrophages (BMDMs) were incubated with CX3CL1 (200 ng/ml) and phenotyped by flow cytometry.
Results: In WT mice, K/BxN serum transfer induced significant allodynia compared to control serum; at 28 days after serum transfer, in DRG, the number of MHCII+ (M1) and MHCII+CD206+ (M1/M2) macrophages was higher than in control serum DRG. However, KO animals developed less severe allodynia than WT and at Day-28 post-serum, the DRG contained less M1 and more MHCII- CD206+ (M2) macrophages than WT. Treatment of BMDMs with CX3CL1 led to polarization of F4/80+ cells towards a M1 phenotype.
Conclusion: CX3CR1-macrophages contribute to allodynia in inflammatory arthritis through cell polarization towards a pro-inflammatory phenotype that sensitises sensory neurons in the DRG and facilitate nociception.

Functional profiling and visualization of fibroblast and epithelial cell dynamics in the influenza virus infected lung

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Influenza A virus (IAV) respiratory infections are a major cause of morbidity and mortality. Stromal cells co-ordinate with immune cells to clear IAV. Few studies have investigated the long-term consequences of IAV infection on lung fibroblasts and epithelial cells. We hypothesise that molecules upregulated by lung stromal cells early following IAV infection persist in order to generate/maintain immune memory.

To address this, we performed RNA-seq on FACS sorted lung epithelial cells and fibroblasts from naïve animals and at early (day 10) and late time points (day 40) following intranasal IAV infection. Biological functions were investigated by flow cytometry. The location of altered stromal and immune cells in the lung was determined using RNAscope and immunohistochemistry.

Analysis of differentially expressed genes (DEGs) demonstrated an enrichment in cell cycle and extracellular matrix genes at day 10. More strikingly, immune related genes were enriched in the DEGs at day 10 and 40; many, including MHCII and CXCL9/10, are regulated by the inflammatory cytokine interferon-γ. SpiB, a transcription factor that regulates genes involved in antigen processing/presentation, was found in epithelial cells in infected mice using RNAscope, but only in airways in close proximity to B220+ immune clusters. Furthermore, immunohistochemistry demonstrated that expression of the immunomodulatory molecule, podoplanin, was limited to cluster adjacent fibroblasts.

Our functional and geographical analysis of the post-IAV lung indicate a prolonged dynamic relationship between immune cells and infection altered stromal cells. These data have important implications for understanding the altered communications between immune and stromal cells during and following subsequent lung infections.

Bacterial LPS, liver stroma and myeloid cell interactions drive immunomodulatory CD14+CD8+T-cells in the human liver

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Background: Despite being bathed in bacterial products like LPS transported from the gut via the portal vein, the liver maintains a state of tolerance, exploited by persistent pathogens and tumours. The cellular basis mediating immune tolerance, yet allowing a rapid switch to immunity, needs to be better defined to deliver successful immunotherapy for liver diseases.

Methods: We analysed the phenotype and function of CD14-expressing CD8+T-cells from human liver and explored their derivation, expansion and LPS-responsiveness in vitro and in vivo.

Results: CD8+T-cells expressing CD14 (and other components of the LPS receptor) were compartmentalised in human liver and preferentially accumulated amongst donor lymphocytes surviving in allografts. CD14+CD8+T-cells were highly activated and proliferative, with constitutive immunomodulatory features at rest (IL-10/IL-2) compared to CD14-CD8+T-cells. They selectively expressed receptors (CXCR4/CD49a/CD49b) supporting retention with the stromal cell network to acquire CD14 from neighbouring macrophages. CD8+T-cells were capable of acquiring CD14/TLR4 and MD-2 from mononuclear phagocytes by actin cytoskeleton-dependent trogocytosis, promoted by stromal cell interaction and LPS. CD14 acquisition conferred upon CD8+T-cells the capacity to bind LPS and respond with a unique functional profile of chemotactic cytokines. Instead, upon TCR-engagement, CD14+CD8+T-cells were poised to mount rapid, anti-viral/anti-tumour function. Therefore, CD14 acquisition by TCR-redirected CD8+T-cells could be exploited to induce superior immunotherapeutic efficacy, with enhanced lysis of hepatoma cells expressing tumour antigen.

Conclusions: A proportion of CD8+T-cells compartmentalised in the liver express CD14/TLR4/MD2, recapitulated in vitro by trogocytosis from mononuclear phagocytes. Bacterial products can therefore fine tune organ-specific immunity by shaping stromal-interacting CD8+T-cells with unique functionality.

Immune contribution to the development of Fibromyalgia

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Fibromyalgia (FMS) is a condition affecting about 2 % of the world population, mostly women. FMS is characterized by chronic widespread pain and pressure hypersensitivity. Moreover, FMS patients also experience cognitive impairment, fatigue, depression, and anxiety. The cause and mechanisms underlying pain and hypersensitivity occurring in fibromyalgia remain unexplained, and there are no effective diagnostic tests or therapeutic strategies available. FMS patient cohort studies revealed a high prevalence of comorbidities related to auto-immune rheumatological conditions, and altered levels of inflammatory cytokines suggesting an immune contribution in the development of FMS. Interestingly, the injection of immunoglobulin G (IgG) purified from FMS patients recapitulated the characteristic mechanical hypersensitivity and reduced spontaneous locomotor activity in mice. In contrast, IgG-depleted patient sera or IgG from healthy donors did not elicit any behavioural changes. In addition to the mechanosensory and locomotor impairments, FMS-injected animals developed an increased sensitivity to noxious cold stimuli and presented a loss of intra-epidermal innervation. We did not detect any differences in the total or subclass IgG concentrations between sera from healthy control (HC) and FMS patients. Dorsal root ganglia (DRG) nociceptive neurons were sensitized in animals treated with FMS immunoglobulin G. Western blot experiments revealed tissue specific binding of FMS IgGs in DRGs. However, FMS IgG did not elicit [Ca2+]i-responses in isolated sensory neurons. Consistent with this lack of direct neuronal activation, immunolabelling experiments revealed that FMS IgGs bind primarily to satellite glial cells in DRGs. These findings suggest an immune cause of fibromyalgia pain mediated by immunoglobulin G.

Dissection and characterisation of stromal cells in persistent neuropathic pain

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Fibroblasts and other stromal cells exist in heterogeneous sub-populations which are integral to both homeostasis and the development of many diseases. Nevertheless, their role in the development of persistent pain has been widely ignored, despite their ability to communicate with and regulate other cell types important for this process, like sensory neurons and immune cells. Here, we dissect and characterise the different populations of fibroblasts and other stromal cells in the mouse sciatic nerve in a well-established model of chronic neuropathic pain: partial sciatic nerve ligation (PSNL). We isolated cells via flow cytometry at early (day 5) and chronic time-points (day 50) and performed 10X single cell RNA sequencing on n=2 male and female mice.
In line with recently published findings, our dataset revealed the presence of several universal fibroblast subsets. The most striking change in cell composition after nerve injury was a reduction in Col15a1+ fibroblasts. At the chronic time point only, we observed a marked increase in numbers for a Notch3+ population, a Fbln1+ population and a Schwann cell population with intermediate expression of Mbp+. These latter Schwann cells were distinct from Mbp negative Schwann cells; specific for Wallerian degeneration, five days after PSNL.
Our results will enable us to generate novel hypotheses about which cell types are implicated in the chronic dysfunction of peripheral nerves after injury. Stromal cells previously have not been considered in detail, and yet, our data clearly show that there are persistent alterations in their phenotype, with potential implications for chronic pain maintenance.

Regulation of intestinal immunity and tissue repair by enteric glia

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Tissue maintenance and repair depend on the integrated activity of multiple cell types. Whereas the contributions of epithelial, immune and stromal cells in intestinal tissue integrity are well understood, the role of intrinsic neuroglia networks remains largely unknown. Here, we uncover pivotal roles of enteric glial cells (EGCs) in intestinal homeostasis, immunity and tissue repair. We demonstrate that infection of mice with *Heligmosomoides polygyrus* leads to enteric gliosis and upregulation of an interferon gamma (IFN-γ) gene signature. IFN-γ-dependent gene modules were also induced in EGCs from inflammatory bowel disease patients. Single-cell transcriptomics of the tunica muscularis (TM) showed that glia-specific abrogation of IFN-γ signaling leads to tissue-wide activation of pro-inflammatory transcriptional programs. In addition, disruption of the IFN-γ-EGC signaling axis enhanced the inflammatory and granulomatous response of the TM to helminths. Mechanistically, we show that upregulation of *Cxcl10* is an early immediate response of EGCs to IFN-γ signaling and provide evidence that this chemokine and the downstream amplification of IFN-γ signaling in the TM are required for a measured inflammatory response to helminths and resolution of granulomatous pathology. Our study demonstrates that IFN-γ signaling in enteric glia is central to intestinal homeostasis and reveals critical roles of the IFN-γ-EGC-Cxcl10 axis in immune response and tissue repair following infectious challenge.

Abstracts: poster presentations

P.01 **Type I interferons mediate antiviral resistance to Zika virus in human microglia and macrophages**

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Type 1 interferons (IFN-α/β) are an important component of the innate immune response to viral infections, yet Zika virus (ZIKV) encodes a range of IFN antagonists, and the role of IFN-α/β in ZIKV immunity in human primary cells remains uncertain. We sought to address this question in microglia and macrophages – cell-types implicated in ZIKV pathogenesis and as viral reservoirs.
We used validated models of human 'microglia-like' cells and macrophages derived from induced pluripotent stem cells (iPSCs), incorporating unique patient-derived iPSCs lacking the human IFN-α/β receptor (IFNAR2), in addition to IFNAR2-/- iPSCs generated by CRISPR/Cas9 gene editing. Cells were challenged with ZIKV and innate IFN induction, ZIKV replication and cytopathic effects were quantified.
Wild-type iPS-microglia and macrophages produced a robust type I interferon response to ZIKV infection which resulted in production of key interferon-stimulated gene products, establishing an antiviral state which restricted replication and protected against cytopathic effects. In contrast, IFNAR2-deficient iPS cells failed to induce ISGs, showed significantly increased ZIKV replication and were highly vulnerable to ZIKV-induced cytopathicity. This phenotype was recapitulated in wild-type cells when they were treated with ruxolitinib, a compound that blocks signalling downstream of IFNAR2.
These results show that IFN-α/β governs resistance of macrophages to ZIKV, suggesting that IFN-α/β may be an important component of immunity to ZIKV in the brain. Further work is required to elucidate the effector mechanisms of this response. These data raise the possibility that molecular defects in IFN-α/β signalling might underlie some extreme clinical phenotypes recognised in ZIKV patients.

P.02 **Autophagy-mediated transition of interleukin signalling is crucial to CD4+ T cell proliferation**

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CD4+ T cells play a central role in adaptive immune function. During ageing, they show impaired proliferation and function, contributing to inadequate responses to infections and vaccines. We discovered that knocking out autophagy in CD4+ T cells mimics this ageing phenotype, demonstrating that autophagy contributes to keeping CD4+ T cells young. Mechanistically, it is unclear what autophagy needs to degrade during CD4+ T cell proliferation selectively. Thus, we created a novel mouse model to investigate this in primary cells based on the proximity labelling technique. It enables us to directly identify the autophagosomal content in most cell types by labelling proteins that neighbours LC3, a molecule enriched in autophagosomes. Among the molecules, we identified with this model is interleukin-7 receptor-α (IL-7Rα). We demonstrate that IL-7Rα is degraded via an LC3-positive compartment during TCR-mediated activation. Since IL-7Rα competes for the γc with IL-2R, accumulation of IL-7Rα on the surface of autophagy-deficient T cells impairs IL-2 signalling, which is indispensable for T cell proliferation. It indicates that autophagy can mediate the transition from cell survival by IL-7 to activation by IL-2. The study further suggests that autophagy induction might be a way to enhance the immune function of aged individuals during vaccination or infections.

P.03 **miR-21-5p regulates sensory neuron to macrophage communication via targeting TGF-β pathway after nerve trauma**

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Background: The expression of non-coding RNA, including miRs is dysregulated in dorsal root ganglia (DRG) after nerve injury and contributes to chronic pain mechanisms. We previously observed miR-21-5p upregulation in exosomal fractions of both cultured DRG stimulated with capsaicin, and nerve-injured DRG. Moreover, miR-21 deletion in sensory neurons protects from development of neuropathic allodynia and macrophage recruitment. Here we aimed to identify miR-21-5p targets in macrophages which may contribute to neuropathic pain development.
Methods: Spared nerve injury (SNI) was used in WT mice and miR-21 conditional KO in sensory neurons (miR-21 cKO) littermates. Lumbar DRGs were isolated and macrophage phenotype was analysed by qRT-PCR and flow cytometry. Peritoneal and bone marrow derived macrophages (BMDM) were transfected with miR-21 mimic or miR-21 antagomir and gene expression measured by qRT-PCR.
Results: At 7 days following SNI, macrophages isolated from DRGs of WT and miR-21 cKO showed a significant alteration of miR-21 known target genes. Overexpression of miR-21-5p in peritoneal macrophages promoted M1-phenotype with up-regulation of TNFα and IL-6. In addition, we found down-regulation of TGF-β1 gene expression and up-regulation of SMAD7 but TβR1, TβR2 and TβR3 were not altered. Down-regulation of miR-21 in BMDMs promoted M2-phenotype with up-regulation of CD206, IL-10, Pparγ and PTEN expression but no changes in arginase, YM1 and TNF-alpha. In addition, TGFβ1 and TβR2 expression were both up-regulated whilst TβR1 and TβR3 were unchanged.
Conclusion: MiR-21-5p silencing in macrophages promotes an M2-phenotype through activation of TGF-β pathway, suggesting that a miR-21 antagomir would polarise macrophages towards an antinociceptive phenotype in neuropathic DRG.

P.04 **Skin Tregs generated early in life are crucial for adult skin homeostasis**

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Skin homeostasis is regulated by both the interaction with peripheral nervous system and immunity. Neuronal activity, innervation and Treg seeding of the skin occur at post-natal day 5 (P5). However, whether the interaction of the two is important for skin homeostasis is unknown. We use Foxp3-DTR transgenic animals to functionally assess neonatal Tregs during stages of postanatal skin development, when the hair follicles become innervated by parasympathetic neurons. We use different depletion regimen: early Treg depletion (eTreg, P6 and P8, co-inciding with neuronal activity), late Treg depletion (lTreg, P10, P12, co-inciding with reduced neuronal activity), and full Treg depletion (fTreg, P6, P8, P10 and P12). Skin defects were assessed using histological techniques, and underlying changes in immune cell composition was profiled by flow cytometry. Additionally, we use whole skin transcriptomics to predict underlying molecular mechanisms. Immediately after fTreg depletion, at P13, effector T cells (Teffs). Whole skin RNA-seq and gene ontology analysis of these skin tissues suggested a disruption of neuronal innervation in the skin. Histological analysis of fTreg-depleted animals at P28 showed visibly less hair and pigmentation. Intriguingly, only eTreg depletion at P6-P8, a phase of high neuronal activity, phenocopied fTreg depletion, whereas lTreg depletion did not. Additionally, eTreg-depleted and fTreg-depleted animals specifically had high number of skin Teffs at P28 suggesting that these cells cause skin abnormalities upon neonatal Treg depletion. Seeding of Tregs early in life may be necessary to prevent precocious activity of Teffs that may in turn disrupt neuronal activity.

P.05 **Acsl4 mutation leads to early and fast progressive hearing loss in mice associated with an inflammatory response within the inner ear**

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Acsl4 (Acyl-CoA synthetase long-chain family member 4) transfers a coenzyme A molecule to free fatty acids to activate the metabolic processing of its lipid targets. It has a high affinity to arachidonic and eicosapentaenoic acids as substrates, and it is a critical enzyme in preventing eicosanoids production. This project is focused on elucidating the effect of Acsl4 mutation and lipid metabolism on auditory function. Acsl4 mutant mice have normal hearing sensitivity at 2 weeks old, but only one week later they dramatically lose hearing sensitivity starting from the high frequencies and by 4 weeks old the thresholds at all frequencies are severely impaired. From the histological analysis of the cochlea, Acsl4 mutants show degeneration of hair cell stereocilia and loss of the sensory hair cell nuclei. The ribbon synapses are reduced in the surviving hair cells. Acsl4 does not appear to regulate transcription of key enzymes involved in eicosanoids production in the arachidonic acid cascade. However, upon flow cytometry analysis at 3 weeks, Acsl4 mutants have an increased number of leukocytes and immune cells involved in the inflammatory response. At 3 weeks we observe a different morphological gradient of the macrophages along the cochlear duct as well as the macrophages in the Acsl4 mutant mice appear to be largely ameboid (activated state), whereas they appear extensively dendritic (resting state) in the controls. Overall, our findings suggest Acsl4 mutation causes an early and fast progressive hearing loss associated with an inflammatory response within the inner ear.

P.06 **The Innate Immune Response during Initiation of Mesothelioma-like Inflammation in the Pleural Space**

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Mesothelioma is a devastating cancer caused by occupational exposure to asbestos fibres. Multi-walled carbon nanotubes (MWCNTs) share a similar fibrous shape, chemical characteristics and bio-persistent properties to asbestos fibres. MWCNTs mimic asbestos exposure leading to mesothelioma-like tumours in murine models.
Fat associated lymphoid clusters (FALCs) are key hubs for the co-ordination of immune responses within serous cavities. FALCs are present within the pericardium & mediastinum but their role in the initiation of mesothelioma has never been investigated.
In this work we aimed to understand the sequence of inflammation precipitating events which occur following arrival of MWCNTs within the pleural space. MWCNTs were injected intra-pleurally in C57BL6/J mice. Early following MWCNT instillation, pleural cavity lavage and adipose tissues were isolated. Immune cell composition, cytokine and chemokine expression and release were determined by flow-cytometry, confocal microscopy & multi-plex array.
MWCNT delivery resulted in increases in Ly6C+ inflammatory macrophages within FALCs of the pericardium but with a striking loss of antigen presentation capacity revealed by significant reduction in MHC-II. The secretome of MWCNT exposed pericardial FALCs was compared with matched serum & pleural fluid from patients with mesothelioma. A strikingly similar secretome to that of human pleural fluid but not serum was found including elevations in CXCL1, CXCL10, CCL2 and IL-6.
Mechanistic studies are addressing the role of CXCL10 in the co-ordination of myeloid cell response to MWCNTs. Complementary in vitro studies are unravelling the combinatorial effects of mesothelioma-relevant chemokines on myeloid cell activation and functionality to improve understanding of immunoregulation during mesothelioma.

P.07 **Lung inflammation causes pericyte loss and vascular remodeling: a role for mast cell activation?**

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Background: The lungs are a highly vascularised organ, however the mechanism by which activation of immune cells, such as mast cells (MCs), modulate blood vessel function remains enigmatic. Typically, blood vessels are lined with an uninterrupted layer of endothelial cells (ECs), facilitating separation between the circulating blood and the interstitial tissue. In addition to ECs, blood vessels are surrounded by structural cells called pericytes, essential to the integrity and development of the vasculature. Despite recent advances, we still do not know how local immune cells can modulate lung pericytes and consequently blood vessel functions.
Method: We utilised neonatal mice model of allergic inflammation, where 3 days old pups were treated with 3 intranasal doses of house dust mite (HDM) per week for 3 weeks. Precision cut lung slices (PCLS) and high-resolution confocal microscopy were employed to investigate the interaction between blood vessel components and immune cells.
Results: Our data indicate that blood vessel density ($CD31^+$ area) is highly heterogeneous and is dependent on the particular lung region. Specifically, vasculature adjacent to large airways and parenchyma exhibited more extensive density compared to the pleura. Interestingly, pericytes ($PDGFR\text{-}b^+$) presented similar heterogeneity with the highest cell number and coverage observed around large airways. During allergic inflammation, this distribution changed dramatically with significant vascular remodelling occurring next to large airways, associated with intense MC activation and immune cell recruitment. This significant reduction in pericyte number and coverage was also observed at distant sites from large airways such as the parenchyma, indicating a potential remote effect.
Conclusion: In summary, our data provide a comprehensive overview of pulmonary pericytes, showing an underestimated heterogeneity with possible important functional roles. In addition, allergic inflammation leads to major changes in the lung vasculature suggesting short- and long-term detrimental effects on lung function.

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