ORAL

Nlrp6 controls cigarette smoke induced lung inflammation via a gut-lung axis and CXCL5 secretion

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Abstract: Tobacco smoke is one of the main factors of chronic obstructive pulmonary disease (COPD). Cigarette smoke components promote repeated aggression of lung barrier and generate airway inflammation. Deciphering the molecular mechanisms of inflammation is of great importance to understand the physiopathology of COPD whose incidence is dramatically increasing.

Using a mouse model of cigarette smoke (CS) exposure, we previously showed that NLRP3 is a key player in lung inflammation. Here we analyzed the role of NLRP6 which expression in intestinal epithelial cells regulates homeostasis, mucus secretion and gut microbiota. However, the role of NLRP6 in lung inflammation remains completely undiscovered.

Exposing wild type (WT) and Nlrp6 deficient (Nlpr6-/-) mice to CS during 4 or 24 days we observed impaired cell recruitment, particularly neutrophils into the bronchoalveolar lavage fluid (BALF) and reduced pro-inflammatory mediators such as CXCL1 or IL-1beta compared to WT. Remodeling factors MMP-9 and MMP-12 in BALF and/or lung of Nlrp6-/- mice are also decreased after CS compared to WT. Importantly, neutrophil recruitment was dependent on Nlrp6 expression in resident cells suggesting a role of lung epithelial cells. Interestingly we observed that epithelial-derived neutrophil chemokine CXCL5, a key chemokine in CS-induced inflammation, accumulated in pulmonary epithelial cells of air- and CS-exposed Nlrp6-/- mice but not in Asc deficient mice demonstrating a role for NLRP6 in CXCL5 secretion independently of the inflammasome. Since CXCL5 also accumulated in Nlrp6-/- intestinal epithelial cells we hypothesized that a gut-lung axis exists. Performing cohousing of WT and Nlrp6-/- mice, we report that reduction of airway inflammation to CS observed in Nlrp6-/- mice was transferred to WT mice after cohousing.

Our results show for the first time that NLRP6 shapes pulmonary inflammation through the control of CXCL5 secretion and gut microbiota independently of the inflammasome.

ORAL

Influenza A virus impairs antimicrobial molecules production in vitro and in vivo, promoting lung bacterial super-infection and IL-1b-mediated inflammation

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Introduction:

Influenza A virus *(IAV)* is an important pathogen, responsible for significant morbidity and mortality world-wide. Although the role of adaptive immunity in controlling IAV opportunistic pathogen is relatively well understood, the function of innate immunity (and that of antimicrobial molecules in particular) is less defined. We therefore set up to study the modulation of antimicrobial production following IAV infection of epithelial cells and that of mice lungs, at homeostasis, or during inflammation (post IL-1ß stimulation).

Methods:

Lung epithelial cell lines (A549, NCI-H292, BEAS-2B) were infected with IAV (with or without IL-1ß stimulation) and the levels of several cytokines (IL-8, CCL-5, IFN ß) and antimicrobial molecules (HBD1, HBD2, HBD3, LL-37, Lcn2, S100A8, S100A9, elafin), were measured by q-PCR and ELISA. In parallel, C57/Bl6 WT mice were infected intra-tracheally (i.t) with 300 pfu of IAV (or PBS as control). 4 days later (at the peak of IAV replication), mice were instilled i.t with 3.10^6 pfu of Ad-elafin (as a source of elafin gene since WT mice are 'natural elafin KO') and with 100 ng of mIL-1ß (or PBS as control). 16hrs later, lung samples, including BALF, were retrieved. BAL cellularity, cytokines and antimicrobials (see above) levels were assessed as above.

Results:

1) Basal expression of antimicrobial molecules was higher in NCI-H292 cells (q-PCR dCT : between -3 and 12) than in A549 or BEAS-2B cells (dCT between 4 and 16), and in all cell lines, HBD1 and elafin levels were the highest (dCT -2 and +2, respectively). When elafin was considered in isolation, IAV significantly up-regulated elafin mRNA in A549 cells (20X), whereas protein induction was less induced (1-2X). By contrast, IL-1ß up-regulated equally A549-derived mRNA and protein (about 1000X), suggesting an IAV-specific post-transcriptional regulation of elafin production.

2) When other mediators than elafin were considered (cytokines and other antimicrobials), IAV induced IL-8, CCL-5 and IFN ß, both at the mRNA and protein levels. Interestingly, IAV significantly induced Lcn2 mRNA (decreased dCT from 0 to -4), but down-regulated Lcn2 protein accumulation (from 4,000 to 1,000 pg/ml).

3) In vivo, IL-1ß exacerbated IAV-induced inflammation, as evidenced by an increase in BAL total cellularity (PBS-Ad-elafin +PBS : 3.105 cells ; PBS-Ad-elafin + IL-1ß : 7.5. 105 ; IAV-Ad-elafin +PBS : 1.2. 106 ; IAV-Ad-elafin + IL-1ß : 1.4. 106 cells) and increased neutrophilia (0 ; 5. 105 ; 5. 105 ; 1.1. 106 , respectively). Strikingly, among all the mediators measured, IAV down-regulated IL-1ß-mediated elafin protein up-regulation (3 fold decrease).

Conclusion:

Taken together, our results show that IAV modulates in vitro and in vivo the expression of antimicrobial molecules such as elafin and Lcn2, at homeostasis, and during an inflammatory episode (mimicked here with IL-1ß stimulation). This may have important implications for bacterial superinfections in viral pneumonias, and we are currently investigating the molecular mechanisms and signalling pathways underlying these processes.

Keywords: Infection-Inflammation

ORAL

The classical NLRP3 inflammasome controls FADD unconventional secretion through microvesicle shedding

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Abstract: FADD (Fas-associated death domain) is a key adaptor molecule involved in numerous physiological processes including cell death, proliferation, innate immunity and inflammation. Therefore, changes in FADD expression have dramatic cellular consequences. In mice and humans, FADD regulation can occur through protein secretion. However, the molecular mechanisms accounting for human FADD secretion were still unknown. Here we report that canonical, non-canonical, but not alternative, NLRP3 inflammasome activation in human monocytes/macrophages induced FADD secretion. NLRP3 inflammasome activation by the bacterial toxin nigericin led to the proinflammatory interleukin-1 beta (IL-1 beta) release and to the induction of cell death by pyroptosis. However, we showed that FADD secretion could occur in absence of increased IL-1 beta release and pyroptosis and, reciprocally, that IL-1 beta release and pyroptosis could occur in absence of FADD secretion. Especially, FADD, but not IL-1 beta, secretion following NLRP3 inflammasome activation required extracellular glucose. Thus, FADD secretion was an active process distinct from unspecific release of proteins during pyroptosis. This FADD secretion process required K+ efflux, NLRP3 sensor, ASC adaptor and CASPASE-1 molecule. Moreover, we identified FADD as a leaderless protein unconventionally secreted through microvesicle shedding, but not exosome release. Finally, we established human soluble FADD as a new marker of joint inflammation in gout and rheumatoid arthritis, two rheumatic diseases involving the NLRP3 inflammasome. Whether soluble FADD could be an actor in these diseases remains to be determined. Nevertheless, our results advance our understanding of the mechanisms contributing to the regulation of the FADD protein expression in human cells.

ORAL

Infection-driven rewiring of fatty acid metabolism helps macrophages contain Mycobacterium tuberculosis growth

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Abstract: Mycobacterium tuberculosis (Mtb), the causative agent of human tuberculosis, has evolved sophisticated strategies to survive in macrophages. In particular, Mtb is believed to hijack host lipid metabolism to stimulate the production of growth-promoting fatty acids (FAs). Using quantitative lipidomics we found that indeed, Mtb infection rewires the FA metabolism of mouse primary macrophages to upregulate saturated FAs and mono-unsaturated FAs. However poly-unsaturated FAs (PUFAs), a subset of FAs that are specific to host cells, were downregulated downstream of essential PUFA precursors. TLR stimulation and Mtb infection had comparable effects on PUFA metabolism, suggesting that PUFAs are downregulated as a response to pathogen pattern recognition. IFN-gamma signaling is known to potentiate the mycobactericidal properties of macrophages through various mechanisms. Interestingly, Mtb infection and IFN-gamma stimulation cooperated to block PUFA biosynthesis, through transcriptional inhibition of rate-limiting biosynthetic enzymes. Further, pharmacological blockade of these enzymes restricted the intracellular growth of Mtb. Altogether, our data reveal a novel mechanism of immune defense involving PUFA metabolism, and highlight the potential importance of PUFAs in regulating infection-mediated inflammation.

ORAL

Specialized proresolving mediators defect and cystic fibrosis airway disease

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Abstract: The acute inflammatory response is host-protective to contain foreign invaders, which is normally self-limited to avoid tissue damage. The resolution of acute inflammation was initially thought to be a passive process and that inflammatory mediators would simply dilute and dissipate to stop the infiltration of leukocytes into the tissues. However, studies performed on mice inflammatory exudates revealed that self-limited acute inflammatory responses involves an active resolution phase designed to restore tissue homeostasis. This resolution phase is carried out by the actions of specialized pro-resolving lipid mediators (SPMs) such as lipoxin, resolvins and maresins. The abnormal production and/or action of SPM is now considered as a pathophysiologic basis associated with widely occurring inflammatory diseases including cystic fibrosis.

In Cystic Fibrosis, the altered hydration of the airway surface (ASL) and mucocilairy clearance that favors chronic bacterial colonization, persistent inflammation and progressive lung destruction is classically explained by ion transport abnormalities directly related to CFTR mutation. However, intrinsic abnormalities of the inflammatory response in CF has been suggested. Several groups including ours have reported an abnormal production of SPMs in the airways of individual with CF that could play a central role in the pathophysiology of CF airway disease. We demonstrated that lipoxin A4 (LXA4) and resolvin D1 (RvD1) regulate airway epithelial function that are altered in CF. These SPMs stimulate CFTR-independent chloride secretion in vitro and in vivo, in human CF airway epithelial cells and in CF mice, and restore the ASL height in human CF airway epithelium. SPMs stimulate epithelial repair and tight junction formation of CF airway epithelium and delay its colonisation by Pseudomonas aeruginosa. SPMs decrease TNF-alpha; induced IL-8 secretion and enhanced the phagocytic and bacterial killing capacity of human CF alveolar macrophages.

Taken together our data provided evidence for a major impact of abnormal lipid metabolism in CF airway disease.

ORAL

ADP-heptose is a newly identified pathogen-associated molecular pattern of Shigella flexneri

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Abstract: During an infection, the detection of pathogens is mediated through the interactions between pathogen-associated molecular patterns (PAMPs) and pathogen recognition receptors. Beta-Heptose 1,7-bisphosphate (BetaHBP), an intermediate of the lipopolysaccharide (LPS) biosynthesis pathway, was recently identified as a bacterial PAMP. It was reported that BetaHBP sensing leads to oligomerization of TIFA proteins, a mechanism controlling NF-kB activation and pro-inflammatory gene expression. Here, we compare the ability of chemically synthesized BetaHBP and Shigella flexneri lysate to induce TIFA oligomerization in epithelial cells. We find that, unlike bacterial lysate, BetaHBP fails to initiate rapid TIFA oligomerization. It only induces delayed signaling, suggesting that BetaHBP must be processed intracellularly to trigger inflammation. Gene deletion and complementation analysis of the LPS biosynthesis pathway revealed that ADP-heptose is the bacterial metabolite responsible for rapid TIFA oligomerization. ADP-heptose sensing occurs down to 10-10 M. During S. flexneri infection, it results in cytokine production, a process dependent on the kinase ALPK1. Altogether, our results rule out a major role of BetaHBP in S. flexneri infection and identify ADP-heptose as a new bacterial PAMP.

ORAL

STING-dependent sensing of self-DNA drives silica-induced lung inflammation

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Abstract: Silica particles induce lung inflammation and fibrosis. We show that stimulator of interferon genes (STING) is essential for silica-induced lung inflammation. In mice, silica induces lung cell death and self-dsDNA release in the bronchoalveolar space that activates STING pathway. Degradation of extracellular self-dsDNA by DNase I inhibits silica-induced STING activation and the downstream type I IFN response. Patients with silicosis have increased circulating dsDNA and CXCL10 in sputum, and patients with fibrotic interstitial lung disease display STING activation and CXCL10 in the lung. In vitro, while mitochondrial dsDNA is sensed by cGAS-STING in dendritic cells, in macrophages extracellular dsDNA activates STING independent of cGAS after silica exposure. These results reveal an essential function of STING-mediated self-dsDNA sensing after silica exposure, and identify DNase I as a potential therapy for silica-induced lung inflammation. Further implication for the control of Mycobacterial tuberculosis infection in the context of silicosis exposure will be discussed.

ORAL

Chromatin dynamics and gene regulation in inflammatory macrophages during skeletal muscle regeneration upon an acute sterile injury

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Skeletal muscle regeneration upon acute sterile injury is a dynamic process that is highly dependent on an *in situ* transition of muscle infiltrating blood monocytes to an inflammatory (Ly6C high) and then to a reparative (Ly6C low) macrophage phenotype. These diverse phenotypes are a combination of differentiation and polarization transcriptional programs that can be transient, stable or permanent, corresponding to cellular activation, polarization and differentiation states, respectively. Here, in order to delineate the temporal order of such transcriptional events we used the cardiotoxin (CTX) acute muscle injury and we systematically profiled chromatin openness (ATAC-seq) and gene expression (RNA-seq) in macrophage populations from injured muscles during the time-course of tissue repair/regeneration. We found a large class (>9.000) of genomic regulatory elements becoming transiently accessible during the first phase of the regeneration process, mainly near genes involved in tissue repair such as cytokines, chemokines, growth factors and stress response mediators. These *de novo* accessible regulatory sites were found to be highly enriched for the AP-1 and MARE (AP-1 motif variation) motifs compared to known macrophage lineage factors' specific motifs. We also identified BACH1, a MARE-binding and heme-regulated transcriptional repressor, as a potential regulatory molecule of macrophage differentiation and polarization. Genetic ablation of Bach1 severely affected inflammatory transcriptional programs and muscle tissue regeneration kinetics upon injury. In addition, cistrome analysis (ChIP-seq) in bone marrow-derived macrophages revealed that BACH1 binds extensively to chromatin, mainly in active and poised enhancers but also to a large class of closed and inactive chromatin regions, suggestive of a bookmarking mode of action. Our data suggest that fine-tuning of transient inflammatory transcriptional programs in macrophages during tissue injury largely depend on AP-1/MARE motif binding transcription factors and that BACH1 is a major regulator of activation and resolution of the inflammatory response.

ORAL

Characterization of the bone micro-architecture of Nlrp-3 knock-out mice.

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Interleukin (IL)-1 is a potent inducer of bone resorption in many inflammatory diseases with bone resorption (Ruscitti P, 2015). Interestingly, IL-1 appears to be involved in bone shaping and physiological remodeling by regulating osteoclasts (Simsa-Maziel S, 2013). Today, the role of Nlrp-3 in the development of bone has never been investigated. In this study, we examined bone micro-architecture in 4 to 16 week-old Nlrp-3 KO and WT mice (males and females) using micro-tomography X-ray (micro-CT) on living animals and histology. At 4 weeks, a significant increase ($p \leq 0,001$) in the total bone mass (BV/TV), the trabecular number (Tb.N), their thickness (Tb.Th), their connection (Tb.Pf) and a disturbed organization of bone structure (SMI) were observed in tibia from NLRP-3 KO mice compared to WT. These differences were sustained up to 8 weeks and then reduced at 12 weeks and 16 weeks. The osteopenic phenotype of 4 to 16 week-old Nlrp-3 KO females was more severe than age-matched. This trend was also observed but with a delay (from 8 weeks) in WT female mice. Using histology, no difference in TRAP staining but a decrease in the number of Von Kossa stained trabeculae were found in femur from 28 days old Nlrp3 KO vs WT mice ($p=0.017$). The quantity of cartilage tissues remaining in the secondary spongiosa was increased in KO Nlrp-3 bone ($p=0.0033$). A reduction of the thickness of the growth plate and of its hypertrophic zone ($p \leq 0.01$), and the decrease of BSP staining ($p<0.001$) in growth plate cells were observed in the Nlrp-3 KO mice. Then, we cultured calvaria osteoblasts (OB) from Nlrp-3 KO vs WT pups. We observed a significant decrease in BSP and Osteocalcin expression ($p \leq 0,01$) at the mRNA and protein levels since two weeks of culture, and a decrease in mineralization (Alzarin Red staining) in OB from KO mice ($p \leq 0,01$). Our study demonstrates that Nlrp-3 KO mice exhibited osteopenic profile in long bones, with reduced trabecular bone mass and disturbed trabecular organization. These abnormalities could be due to impaired osteoblasts mineralization.

ORAL

Spatio-temporal study of inflammation during the early phase of myocardial ischemia-reperfusion

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Abstract: Cardiovascular diseases remain the leading cause of mortality in the world. Despite the increasing effectiveness of the management of patients with myocardial infarction, mortality and morbidity for this pathology remain too high. It is now well known that inflammation contributes to the pathology of myocardial infarction, making it a new therapeutic target. Some clinical trials have been conducted targeting the chronic phase of inflammation and some results seem promising but do not show any improvement in the clinical outcomes. Our proposal focuses on the early phase (the first 24 hours) of myocardial infarction since these early events are the driving force of the later events. Following myocardial infarction, immune cells are recruited to the heart in different waves. Currently studied by immunohistochemistry between 24H and 7 days of reperfusion on heart slices, we propose in our project to decipher the immune cells infiltration in 3 dimensions in the whole organ throughout the early phase of reperfusion. The recent development of clearing methods in neurosciences opens new insights through giving access to high spatially resolved whole organ imaging. Indeed, tissue fixation followed by lipids removing increases the scattering of light travelling through the tissue in microscopy. We have adapted this technique for the heart, and it opens new insight in myocardial infarction. Briefly, using X-Clarity system, mice hearts were clarified after ischemia-reperfusion sequences, immunolabelled with antibodies recognizing the immune cells, especially macrophages. Then, hearts were imaged by confocal microscopy and light-sheet imaging systems to visualize and quantify the infiltration of macrophages according the time course of the early phase of myocardial infarction in 3D.

ORAL

Insights into mechanisms of innate immunity: specific interactions measured by AFM between peroxiredoxin-5 and Toll-like receptor 4

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Abstract: Inflammation is a pathophysiological response of innate immunity to infection or tissue injury. This response can be triggered by endogenous molecules released from damaged cells, known as damage-associated molecular patterns (DAMPs). Members of the superfamily of peroxidases named peroxiredoxins (PRDXs) have been reported to act as DAMPs in inflammatory processes such as post-ischemic inflammation. One key question is to understand how extracellular PRDXs are able to activate pattern recognition receptors such as Toll-like receptors (TLRs) to induce inflammation. These mechanisms are of great importance, being implicated in the pathogenesis of many diseases. Here, we used atomic force microscopy (AFM), combining complementary acellular and cellular approaches, to investigate how human PRDX5 can trigger a proinflammatory response through TLR4 activation. Force-distance (FD) curve-based AFM is a recently established method that simultaneously allows imaging at high resolution and quantifying mechanical properties as well as biological, chemical and physical interactions. Single-molecule experiments using FD-based AFM demonstrated that PRDX5 specifically binds to TLR4. We showed that this interaction is also established on macrophage-differentiated THP-1 cells and combination with fluorescence allowed us to demonstrate that it is specifically mediated by human TLR4 on transfected TLR4-YFP CHO cells. Moreover, our results revealed that PRDX5 binding induces a cellular mechanoresponse, i.e. a stiffening of the cell and a change in its morphology. These findings provide new insights into the first key step of the inflammatory pathways and warrant further exploration as a potential novel therapeutic target for inflammatory diseases.

ORAL

Viscoelastic properties of neutrophils during phagocytosis

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Phagocytosis is one of the first steps of an immune response which can lead to a local inflammation. To phagocyte, a **neutrophil** needs to surround its target with a phagocytic cup. While the cell spreads on the target, it reorganizes its cytoskeleton and exerts forces on the target. Force generation correlates with an increase in cell stiffness. However, cells being viscoelastic (they are described by both viscosity and stiffness), a complete understanding of mechanical properties requires measuring changes in viscous properties, which are yet unknown. The observed increase in cell stiffness during phagocytosis suggests that viscoelastic changes might be important to perform phagocytosis. Yet, what specific cytoskeletal-associated molecules are key to these events is still unclear. We further ask if the mechanical state of the cell before ongoing phagocytosis can influence the outcome of the process. This could be relevant in the context of inflammation, as immune cells in an **inflamed environment are mechanically different**. Our goal is thus to measure how inflammation can affect viscoelastic properties of neutrophils before and during phagocytosis.

We designed a single-cell rheometer to quantify changes in viscoelastic properties of non-adherent cells. This micropipette-based setup combines morphological and mechanical measurements. We put in contact a neutrophil with an antibody-coated polystyrene bead and apply a compressive force to the cell using a flexible micropipette used as a cantilever. Once a desired level of force is reached, the cantilever oscillates to impose oscillatory variations in force level through an automated feedback. We monitor the evolution of the **viscoelastic properties** of the cell while it spreads on the bead by measuring the elastic part (K') and the viscous part (K") of its so-called complex stiffness.

We show that during phagocytosis, both **stiffness and viscous properties increase** ten and four folds, respectively. These changes in viscoelastic properties could explain the spreading dynamics and help understanding the role of cytoskeletal rearrangements in establishing a phagocytic cup. Our on-going work aims at quantifying how these mechanical properties can be changed by inflammation-mediators such as TNFα.

POSTER n°1

Effect of stem cells on tissue regeneration via macrophages M2d

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Abstract: Angiogenesis is very important during tissue regeneration; it is regulated by different growth factors and cytokines. Cytokines are secreted by many cell populations; the predominant producers are macrophages and helper T cells. The identification of different subpopulations of macrophages would allow a better understanding of the mechanisms of angiogenesis and therapies for tissue repair. Local injections of adipose tissue-derived stem cells (ASCs) have been shown to reduce inflammation and improve tissue regeneration. However, the mechanisms involved need to be deepened.
We characterized the different subpopulations of macrophages of different muscles (76 days after local irradiation with 50 Gy gamma, minipig animal model) by immunolabeling and in situ hybridization in animals (irradiated and treated with ASC and in our study, we found mainly M2 and some M1 macrophages in the IRR + ASC muscle Only a few M2b macrophages were observed in the IRR muscle. The different types of macrophages express a panel of different cytokines assigning them different functions We analyzed the expression of the cytokines of the different macrophages present in the muscle IRR and (IRR + ASC), and we showed that the M2d macrophages are the most abundant in the IRR + ASC samples. We have shown by in situ hybridization that M2d macrophages express TNF-alpha; and TGF-Beta. These two cytokines are very important in inducing the development of blood vessels. Our working hypothesis is that stem cells play role in the appearance of M2d macrophages. The cytokines expressed by these macrophages would induce angiogenesis to enhance tissue regeneration.

POSTER n°2

Investigating the kinetics of immune cell metabolism during MVA-induced inflammatory response

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Abstract: Immune responses are largely regulated by changes in the metabolic activities of various cell types including monocyte-derived cells, dendritic cells and T cells. Metabolic changes in myeloid cells are, for example, essential to regulate cytokine and chemokine production. T cells also undergo a profound metabolic switch during activation, a feature essential for proper clonal expansion and acquisition of effector functions. However, how vaccine candidates impact on the metabolic activities of immune cells and how metabolic changes influence the vaccine efficiency is largely unknown.

A potential regulator of cell metabolism is nitric oxide (NO) produced by myeloid and stromal cells. NO has pleiotropic effects including the ability to dampen mitochondrial respiration (OXPHOS). Modified vaccinia virus Ankara (MVA) is an attractive viral vector that is being currently tested for vaccination against cancer and infectious diseases. MVA is characterised by a massive recruitment of monocyte-derived cells into the draining lymph nodes (dLN). We hypothesise that MVA induces a profound metabolic reprogramming in the dLN and that subsequently, NO produced by recruited myeloid cells further regulates immune cell metabolic activity.

Preliminary results suggest that interfering with NO activity during vaccination improves the generation of antigen-specific T cell response. To dissect how the MVA candidate vaccine act on immune cell metabolism and assess the specific contribution of NO in the regulation of the immune response, we implemented a new fluorescent reporter for cell metabolism, PercevalHR. We characterised the fluorescent reporter to detect metabolic fluxes in monocyte-derived cells and T cells in real-time with single-cell resolution in vitro. We plan to apply PercevalHR in vivo and measure metabolic changes spatio-temporally and draw a map of NO activity during MVA-induced inflammatory response and determine how NO shapes the metabolic activity of immune cells in the complex infection site. Overall, this project will represent a major progress in the immunometabolism field, contributing to better understand the interplay between changes in cellular metabolism and vaccine efficiency.

POSTER n°3

Sensitivity of B220$^+$ T cells to P2X7 receptor-mediated cellular functions are dependent on membrane cholesterol content

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P2X7 purinergic receptor (P2X7R) plays a central role in both innate and adaptive immunity. Recently, we reported that ATP-mediated cellular activities in CD4$^+$ and CD8$^+$ T cells are not dependent on the levels of P2X7R membrane expression, but depend on their stage of activation/differentiation. Moreover, we have reported that pathogenic B220$^+$ CD4$^-$CD8$^-$ T cells that accumulate in secondary lymphoid organs of auto-immune MRL/*lpr* mice during ageing, or normal B220$^+$ T cells at the preapoptotic stage have lost P2X7R membrane expression and ATP sensitivity, suggesting that the ATP/P2X7R pathway could contribute with the Fas pathway to T-cell homeostasis and autoimmunity. We show herein that although both EL4 and L1210 T-cell lines express high levels of P2X7R at the plasma membrane, only EL-4 cells display high ATP sensitivity. Since we shown previously that high level of B220 is expressed on L1210 T cells, we suggest that the protein tyrosine phosphatase B220 (CD45RABC isoform) could impair the functionality of P2X7R. Interestingly, it has been shown that ATP-mediated P2X7R activation is dependent on membrane cholesterol levels. Therefore, we have hypothesized that cholesterol membrane content could be responsible of the loss of ATP sensitivity in B220$^+$ T cells (either normal or pathogenic). Using Filipin Fluorescence staining of free cholesterol, we found that B220$^+$ L1210 T cells express higher levels of membrane cholesterol than EL-4 T cells. To get insight on the role of cholesterol content in the sensitivity of B220$^+$ T cells to ATP, we have analyzed P2X7R-mediated cellular functions (calcium influx, pore formation, cell death) in B220$^+$ L1210 T cells after cholesterol depletion with Methyl-Beta-Cyclodextrin. Interestingly, we found that cholesterol depletion significantly reduced the levels of B220 membrane expression and restores P2X7- mediated cellular functions in L1210 T cells. Our data reveal a strong correlation between membrane levels of phosphatase B220, cholesterol content and P2X7-mediated cellular functions.

POSTER n°4

Nod-like receptor pyrin domain-containing protein NLRP6 interacts with casein kinase 2 and regulates cycling of stem cells in intestinal crypts.

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Abstract: We have previously established that the inflammasome component NLRP6 contributes to proper gut wound healing and protects mice from colitis and colorectal cancer. However, the cellular mechanism of this protection is not understood. Herein, we identify the regulatory beta subunit of the casein kinase 2 (Csnk2b) as an interacting partner of NLRP6 that we found to be primarily expressed in stem cells. This interaction increases after induction of SSBs by a H2O2 treatment in models of human cancer intestinal cells, and we have previously shown that cell growth is stopped by the DNA repair process controlled by CK2. Equally of importance our data demonstrates that the proliferation rate was enhanced by about 50 percent in HEK293 cells overexpressing NLRP6 despite no difference in a specific cell cycle stage and apoptosis. Conversely, a 30 percent lowered proliferation rate of primary mouse embryonic fibroblasts (MEF) coincided with an impaired autophagy in the absence of NLRP6. Altogether, these data suggest that NLRP6 interacts by CK2 and could control cycling of stem cell.

POSTER n°5

Nod-like receptor pyrin domain-containing protein NLRP6: evidences of a new short isoform and its involvement in senescence of epithelial cells

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Abstract: Cellular senescence is a stress-induced state characterized by a robust cell cycle arrest, an apoptosis resistance, a pro-inflammatory cytokines secretion, and a persistence of unrepaired DNA damages. During aging, senescent cells accumulate in most tissues and organs and contribute to most age-associated disorders and pathologies. In the gastro-intestinal tract, the tissue capacity to repair injuries declines with age, potentially leading to chronic inflammatory diseases and to colorectal cancer. However, nothing is known about the contribution of epithelial cell senescence in the wound healing of the intestinal epithelium. We have previously established that the inflammasome component NLRP6 contributes to proper gut wound healing and protects mice from colitis and colorectal cancer. Therefore, we aim in this project to investigate a potential role of NLRP6 in intestinal epithelial cell senescence and how this interferes with the efficacy and accuracy of gut wound healing.
We first overexpressed NLRP6 in three colorectal cancer cell lines. We showed an increase in cell death following NLRP6 overexpression, but no senescence induction. During these experiments, we have witnessed that all cell lines commonly express a possible short NLRP6 isoform (sNLRP6). According to a gene prediction in NCBI database supported by EST evidence and our preliminary results, sNLRP6 could be a NLRP6 isoform devoid of the N-terminal-Pyrin domain, being therefore unable to initiate the canonical ASC-caspase1 inflammasome assembly. Interestingly, sNLRP6 expression increases at senescence of human primary keratinocytes (used as a model of normal epithelial cell). We have previously shown that senescence in these cells results from accumulation of DNA single-strand breaks (SSBs), because of a default of phosphorylation of the scaffold protein XRCC1 by the serine-threonine kinase CSNK2, a primordial step in the SSB repair pathway. Therefore, we hypothesized that sNLRP6 could act on CSNK2 activity. We demonstrate by co-immunoprecipitation that sNLRP6 interacts with CSNK2. This interaction increases at senescence and after induction of SSBs by a H_2O_2 treatment. These results suggest that this new short NLRP6 isoform could play a role in age-associated colitis and colorectal cancer by interfering with the repair of DNA damages of oxidative origin, thereby favoring intestinal epithelial cell senescence.

POSTER n°6

Tissue nonspecific alkaline phosphatase (TNAP) is an anti-inflammatory ectonucleotidase

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Abstract: TNAP is a GPI-anchored protein whose main function is to allow bone mineralization. This function is achieved by the hydrolysis of the mineralization inhibitor inorganic pyrophosphate (PPi), which is mainly generated from extracellularly released ATP by the ectonucleotidase pyrophosphatase phosphodiesterase 1 (NPP1). We hypothesized that TNAP is also able to dephosphorylate ATP and participate to the resolution of ATP-associated inflammation. In this hypothetic model, TNAP would participate with CD39 and NPP1 to the hydrolysis of ATP, and with CD73 to that of AMP. Our hypothesis relied on the fact that patients with hypophosphatasia (genetic TNAP deficiency) not only have hypomineralized bones, but also suffer from chronic recurrent multifocal osteomyelitis (CRMO). RT-qPCR of bones of 7-day-old Tnap+/- mice revealed increased levels of Il-1 Beta; (60%) and Il-6 (250%) and decreased levels of anti-inflammatory Il-10 (50%) as compared with Tnap+/+ mice, suggesting CRMO. In mouse primary osteoblasts and growth plate chondrocytes, Tnap was more than 10-fold more expressed than Npp1, Cd39 and Cd73, suggesting that it participates to ATP and AMP hydrolysis. TNAP inhibition with MLS-0038949 in growth plate chondrocytes significantly dropped the dephosphorylation of exogenously added ATP and AMP (Pi quantification with malachite green) and led to higher levels of ATP released by the cells (luciferase activity). These effects on ATP and AMP dephosphorylation were probably physiologically relevant because TNAP inhibition also led to increased IL-6 secretion. Finally, treatment of both osteoblasts and growth plate chondrocytes with IL-1beta; dramatically reduced TNAP expression but increased the levels of CD73, suggesting that in inflamed bones, CD73 is the main nucleotidase dephosphorylating AMP. These inflammatory effects are particularly worrying in the context of hypophosphatasia, where a vicious cycle may exist, during which TNAP deficiency triggers inflammation, which in return exacerbates TNAP loss of function. In conclusion, our results demonstrate that TNAP is an anti-inflammatory ectonucleotidase.

POSTER n°7

BAFF is involved in acute inflammation induced by cigarette smoke exposure in mice

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Abstract: Introduction. The mechanisms of chronic Obstructive Pulmonary Disease (COPD), a progressive pulmonary disease without effective treatment, are not understood and mainly due to cigarette smoke. We investigated the contribution of the B-cell activating factor (BAFF), a TNF family member implicated in the generation of lymphoid follicles which are associated with the severity of COPD. Moreover, BAFF is implicated in the regulation of pathogenic IL-17 producing cells in autoimmune diseases. We showed previously a critical role of BAFF in experimental lung fibrosis by amplifying the IL-17A effect. Here we studied the role of BAFF in the development of inflammation caused by acute cigarette smoke (CS) exposure.
Methods. Wild-type mice (WT), BAFF (BAFF-/-) and IL-17RA (IL-17RA-/-) deficient mice were exposed to the smoke of 4 cigarettes (3R4F), 3 times a day during 4 days. Mice were sacrificed 16 hours after the last exposure. Broncho-alveolar lavage fluids (BALF) were done and cell infiltration was determined. Content of pro-inflammatory cytokines and remodeling factors were measured in BALF and lung homogenates. BAFF immunostaining was done on BALF cells from WT mice exposed to CS.
Results. We observed that BAFF expression is greatly increased after CS exposure in WT. In BAFF deficient mice, macrophages and neutrophils recruitment in the BALF is greatly reduced as the expression of IL- 1beta; and remodeling factors in lung tissue, in particular the expression of MMP-9 protease.
IL-17RA-/- mice exhibit a decrease BAFF rate suggesting the involvement of IL-17A pathway in BAFF production. The instillation of recombinant BAFF in WT mice, in addition to CS exposure, increases the recruitment and activation of neutrophils. The instillation of recombinant BAFF in WT mice, in addition to CS exposure, increases the recruitment and activation of neutrophils. BAFF immunostaining on BALF cells reveals that BAFF is mainly expressed by neutrophils.
Conclusions. Our data therefore support a role for BAFF in the establishment of pulmonary inflammation induced by CS exposure at the interface between innate and adaptive immunity, through a crosstalk between BAFF and IL-17A. Moreover, BAFF is mainly expressed by neutrophils after CS.

POSTER n°8

New *in vitro* models of mucus-secreting and inflammatory airway epithelium

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Chronic obstructive pulmonary disease (COPD) is a lung disease characterized by chronic inflammation and mucus hypersecretion. Currently, curative treatment does not exist, and some patients develop resistance to available drugs. Gene therapy could be a novel approach to treat the disease and improve patients' quality of life. However, the effectiveness of gene vectors in the airways is decreased by the presence of mucus that acts as a barrier. The aim of this study was to develop an *in vitro* model of mucus-secreting and inflammatory airway epithelium for testing efficacy of new drugs including mucolytic gene therapy vectors.

We used three airway epithelial lines (A549, Calu-3 and NCI-H292) that were stimulated with different concentrations of CSE (Cigarette Smoke Extract) alone or in association with LPS (lipopolysaccharides), for 24 hours. NCI-H292 and Calu-3 cells are also co-cultured in the same conditions. Then, we evaluated MUC5AC, IL-8/CXCL8, GROα/CXCL1 and MCP- 1/CCL2 gene expression by RT-qPCR and the production of cytokines by ELISA.

CSE alone or with LPS did not impact MUC5AC gene expression, in A549 cells. In contrast, LPS (0.1 µg/mL) increased IL-8/CXCL8, GROα/CXCL1 and MCP-1/CCL2 release. When CSE was associated with LPS, there was an additive effect on cytokine release.

Regarding Calu-3 cells, treatment with CSE, LPS, or both, did not affect MUC5AC gene expression, neither cytokine secretions.

For NCI-H292 cells, CSE alone increased MUC5AC gene expression and an additive effect was observed when CSE was associated with LPS. LPS at low concentrations triggered some IL-8/CXCL8 release, which was more important when LPS was associated with CSE; but not for GROα/CXCL1. NCI-H292 cells did not release MCP-1/CCL2.

In the NCI-H292 and Calu-3 co-culture, CSE and LPS increased MUC5AC gene expression, but CSE did not affect cytokine secretion. LPS alone increased IL-8/CXCL8 secretion, but not GROα/CXCL1 or MCP-1/CCL2.

Our results showed that NCI-H292 cells, alone or in co-culture with Calu-3, appear as the best model to evaluate the efficacy of gene vectors, as there are able to produce mucins and cytokines, after CSE and LPS exposure. Nonetheless, the co-culture could allow obtaining production of a greater panel of cytokines.

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POSTER n°9

ADP-heptose is a newly identified pathogen-associated molecular pattern of Shigella flexneri

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Abstract: During an infection, the detection of pathogens is mediated through the interactions between pathogen-associated molecular patterns (PAMPs) and pathogen recognition receptors. Beta-Heptose 1,7- bisphosphate (Beta-HBP), an intermediate of the lipopolysaccharide(LPS) biosynthesis pathway, was recently identified as a bacterial PAMP. It was reported that Beta-HBP sensing leads to oligomerization of TIFA proteins, a mechanism controlling NF-kB activation and pro-inflammatory gene expression. Here, we compare the ability of chemically synthesized Beta-HBP and Shigella flexneri lysate to induce TIFA oligomerization in epithelial cells. We find that, unlike bacterial lysate, Beta-HBP fails to initiate rapid TIFA oligomerization. It only induces delayed signaling, suggesting that Beta-HBP must be processed intracellularly to trigger inflammation. Gene deletion and complementation analysis of the LPS biosynthesis pathway revealed that ADP-heptose is the bacterial metabolite responsible for rapid TIFA oligomerization. ADP-heptose sensing occurs down to 10-10 M.During S. flexneri infection, it results in cytokine production, a process dependent on the kinase ALPK1. Altogether, our results rule out a major role of Beta-HBP in S. flexneri infection and identify ADP-heptose as a new bacterial PAMP.

POSTER n°10

Innate Immune Response of Primary Human Keratinocytes to West Nile Virus Infection and Its Modulation by Mosquito Saliva.

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Abstract: West Nile Virus (WNV) is a flavivirus involved in many human infections worldwide. This arthropod-borne virus is directly co-inoculated with mosquito saliva through the epidermis and the dermis during blood meal. WNV starts replicating in the skin before migrating to the draining lymph node, leading to widespread viremia and in some cases to neurological symptoms. Skin is a complex organ composed of different cell types that together perform essential functions such as pathogen sensing, barrier maintenance and immunity. Keratinocytes, which represent 90% of the cells of the epidermis, are the organism's first line of defense, initiating innate immune response by recognizing pathogens through their pattern recognition receptors. Although WNV was previously known to replicate in human primary keratinocytes, the induced inflammatory response remains unknown. The aim of this study was first to characterize the inflammatory response of human primary keratinocytes to WNV infection and then, to assess the potential role of co-inoculated mosquito saliva on the keratinocyte immune response and viral replication. A type I and III interferon inflammatory response associated with an increase of IRF7 but not IRF3 mRNA expression, and dependent on infectious dose, was observed during keratinocyte infection with WNV. Expression of several interferon-stimulated gene mRNA was also increased at 24 h post- infection (p.i.); they included CXCL10 and interferon-induced proteins with tetratricopeptide repeats (IFIT)-2 sustained up until 48 h p.i. Moreover, WNV infection of keratinocyte resulted in a significant increase of pro- inflammatory cytokines (TNF alpha, IL-6) and various chemokines (CXCL1, CXCL2, CXCL8 and CCL20) expression. The addition of Aedes aegypti or Culex quinquefasciatus mosquito saliva, two vectors of WNV infection, to infected keratinocytes led to a decrease of inflammatory response at 24 h p.i. However, only Ae. Aegypti saliva adjunction induced modulation of viral replication. In conclusion, this work describes for the first time the inflammatory response of human primary keratinocytes to WNV infection and its modulation in presence of vector mosquito saliva. The effects of mosquito saliva assessed in this work could be involved in the early steps of WNV replication in skin promoting viral spread through the body.

POSTER n°11

Comparison of platforms quantitating fg/mL biomarkers using bead-based and planar technology: the benchtop readers SR-X™, SP-X™, and the fully automated analyzer HD-1

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Abstract: Digital ELISA (Enzyme Linked Immunosorbent Assay) based on single molecule arrays (Simoa) has improved sensitivity of traditional ELISA from picomolar (10-12 M) to femtomolar (10-15 M), increasing the quality and quantity of biomarkers that can be measured for health and disease. Digital ELISA counts signal generated from single immunocomplexes formed on superparamagnetic beads confined in arrays of femtoliter- sized wells in which fluorescent molecules are highly concentrated. We have commercialized digital ELISA in a fully-automated instrument (Simoa HD- Analyzer), ideal for use in pharmaceutical companies, drug discovery, clinical research and other areas necessitating full automation and high throughput. Based on the same technology, we have also commercialized the SR-X benchtop reader, with a smaller footprint and more flexible workflow. Operators prepare assays in microtiter plates at the bench in a semi-automated format similar to traditional ELISA, with the notable exception that plates are preserved by drying after assay completion, and can be read immediately or the next day. We have recently launched another bench top instrument, SP-X imaging system. With a workflow similar to SR-X, SP-X offer comparable sensitivity with higher level of multiplexing using planar-based technology.

POSTER n°12

Bipolar effects of cigarette smoke in macrophages and monocytes from chronic obstructive pulmonary disease patients

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Introduction: Cigarette smoke (CS) is the main risk factor of chronic obstructive pulmonary disease (COPD). Macrophages are an important source of pro-inflammatory anti-inflammatory mediators that play an important role in COPD. Much of the knowledge of CS and macrophages has been derived from animal models, cell line and gene expression. However little is known about the true role human macrophages phenotype and its functionality. The aim of this study was to investigate which cytokines are involved in human macrophages/monocytes exposed to CS and compared to those obtained from COPD patients.
Methods: Cigarette smoke extract (CSE) was prepared using Kentucky 2R1 cigarettes in contact with 20 mL medium. Monocyte from 30 patients and 20 donors were obtained from blood. Monocytes were incubated with GM-CSF (50ng/ml) to obtain macrophages. Cells were treated with CSE (2%-10%) alone or in combination with LPS (0.1µg/ml) and IL-4 (10ng/ml).
Result: CSE did not appear affect M1 cytokines such as TNF-α, CCL2/MCP-1 and IL-6, but CSE increase production of Gro-a/CXCL1 and IL-8/CXCL8. In contrast, CSE suppressed the production of IL-6 and Gro-a/CXCL1 induced by LPS. CSE also had an additive effect in IL-8/CXCL8 and CCL-2/MCP-1 release when associated to LPS. Regarding M2 cytokines, CSE seems had not effect in IL-10 and PARC/CCL-18 release. Moreover, it induced TARC/CCL- 17 and CCL2/MDC production. However, the association of CSE and IL-4 induced release of M2 cytokines like TARC/CCL17 and PARC/CCL8 when compared to IL-4 alone. Macrophages had also a worsening of uptake capacity of microspheres after CSE exposure. Regarding the data from COPD patients, the monocytes treated with LPS showed a low levels of IL-8 / CXCL8 and Gro-α/CXCL1 release when compared to monocytes from healthy donors. However, monocytes from COPD treated with IL-4 showed high levels of TARC/CCL17 an MDC/CCL-22.
Conclusion: These data showed that CSE could modify macrophages profile, suggesting a phenotype M2 “like”. Furthermore, there similar change of phenotype the monocytes from COPD patients. We suggest that the monocytes of COPD patients can have already functionality predisposed to an M2 phenotype that can contributes to progression of COPD.
Acknowledgments: CAPES.

POSTER n°13

A novel mouse model of autoimmune and lymphoproliferative syndrome reveals a major role of purinergic receptor P2X7 in T cell homeostasis

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The severe autoimmune diseases developed by MRL/*lpr* mice depend on the interaction between the *lpr* mutation of the Fas gene and additional genes of pathologic importance still remain to be discovered in the MRL genetic background. In contrast, C57BL/6 (B6) mice bearing the *lpr* mutation develop only a mild lymphoproliferation and a limited lupus-like. Previously, we have reported that pathogenic $B220^+$ $CD4^-CD8^-$ (DN) T cells that accumulate in secondary lymphoid organs of MRL/*lpr* mice during ageing have lost P2X7R membrane expression and ATP sensitivity, suggesting that the ATP/P2X7R pathway could contribute with the Fas pathway to T-cell homeostasis and autoimmunity. Herein, we have generated and characterized a novel mouse strain (called B6/*lpr*-P2X7KO) that harbors homozygous *lpr* mutation and genetic inactivation of P2X7R in the B6 genetic background. In striking contrast to the mild or absent phenotype observed in B6/*lpr* or B6-P2X7KO mice, B6/*lpr*-P2X7KO mice, especially females, exhibit a severe phenotype characterized by massive hepatomegaly, splenomegaly, and lymphadenopathies. This lymphoproliferative syndrome is caused by the accumulation of FasL- expressing $B220^+$ DN T cells, which mainly originate from $CD45RB^{high}CD44^{high}$ effector/memory $CD8^+$ T cells. Moreover, sera from B6/*lpr*-P2X7KO mice contain high levels of IgG anti-dsDNA auto-antibodies and rheumatoid factor as well as chemokines and cytokines. B6/*lpr*-P2X7KO mice have a shortened lifespan, but that remains significantly longer than MRL/*lpr* mice. To conclude, the B6/*lpr*-P2X7KO strain reproduces many aspects of the autoimmune disease of the MRL/*lpr* strain that suggest a synergistic role of the Fas/FasL and ATP/P2X7R pathways in T-cell homeostasis and consequently in autoimmunity.

POSTER n°14

Functional characterization of the cGAS -STING pathway in CD4+T cells

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Abstract: While the response of innate immune sensors to pathogens is well understood in myeloid cells that constitute a first line of defense, the functionality of these pathways in cells of the adaptive immune system is poorly understood. In humans, CD4+ T cells are the primary targets of HIV. While the cGAS-STING pathway is critical for sensing HIV in myeloid cells, its functionality in CD4+ T cells remains ill defined.
Immune response of activated CD4+ T cells and monocyte-derived dendritic cells (DCs) from healthy donors stimulated with synthetic ligands (DNA, cGAMP) was monitored by IFN measurement, CD86 and SIGLEC1 expression.
We have recently demonstrated that active STING has a non-canonical function of inhibiting proliferation in CD4+T cells that is independent of IFN-I and known interactors TBK1 and IRF3. Now, using multiple delivery methods of agonists, we find that induction of IFN by both cGAS and STING activation is severely compromised in donor-matched CD4+ T cells over DCs. Intrinsic STING activity is one of the factors limiting the IFN response in CD4+T cells.
Our findings provide an essential characterization of the cGAS-STING pathway in CD4+ T cells, and reveal a largely disabled state, which may benefit HIV replication.

POSTER n°15

Ozone-induced chronic epithelial tissue injury causes emphysema and lung fibrosis

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Abstract: Air pollution associated with ozone exposure represents is a major cause of respiratory disease in man. In mice, a single ozone exposure causes lung injury with disruption of the respiratory barrier and inflammation, which is interleukin-1 dependent. Here we demonstrate that chronic ozone exposure (1.5ppm, 2h, twice weekly) causes chronic epithelial injury with disruption of respiratory barrier, chronic inflammation, airway hyperreactivity, emphysema and lung fibrosis, which are IL-1α dependent signaling via IL-1R adaptor protein MyD88. Importantly, epithelial cell signaling is critical, since deletion of MyD88 in lung type I alveolar epithelial cells reduced ozone-induced inflammation. In conclusion, the alarmin IL-1alpha; released upon ozone-induced epithelial damage and inflammation is mediated by IL-1 alpha, which may represent a therapeutic target to attenuate ozone-induced lung inflammation and hyperreactivity.

POSTER n°16

STING signaling pathway regulates idiopathic pulmonary fibrosis

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Abstract: Idiopathic pulmonary fibrosis (IPF) is the most common and severe type of interstitial lung disease for which current treatments display limited efficacy. Using a mouse model of this pathology, we obtained preliminary results showing significant contributions for the intracellular DNA sensor stimulator of interferon genes (STING), a protein known to lead to type I or type III interferon (IFN) production. Our data revealed that as compared to bleomycin (BLM) instilled C57BL/6 wild type (WT) mice, STING deficient mice display exacerbated pulmonary fibrosis, characterized by higher bodyweight loss, increased collagen deposition in the lungs and excessive expression of the important remodeling factors MMP-9 and TIMP-1. However, type I IFN receptor deficient mice displayed similar phenotype as WT mice, indicating that STING contribution is occurring independently of type I IFN signaling. In contrast, mice deficient for type III IFN (also known as IL-28) receptor showed similar phenotype as STING deficient mice, and IL-28 production is decreased in BLM treated STING deficient mice. We are currently attempting to comprehensively address the unknown contribution of self-nucleic acid recognition and its potential link with type III IFN signaling pathways to limit the pathological development leading to IPF.