International Symposium

Neutrophil 2018

Québec City, June 2-5, 2018

www.theneutrophil.com

Program and Abstracts

Neutrophil 2018

Québec City, June 2-5 2018

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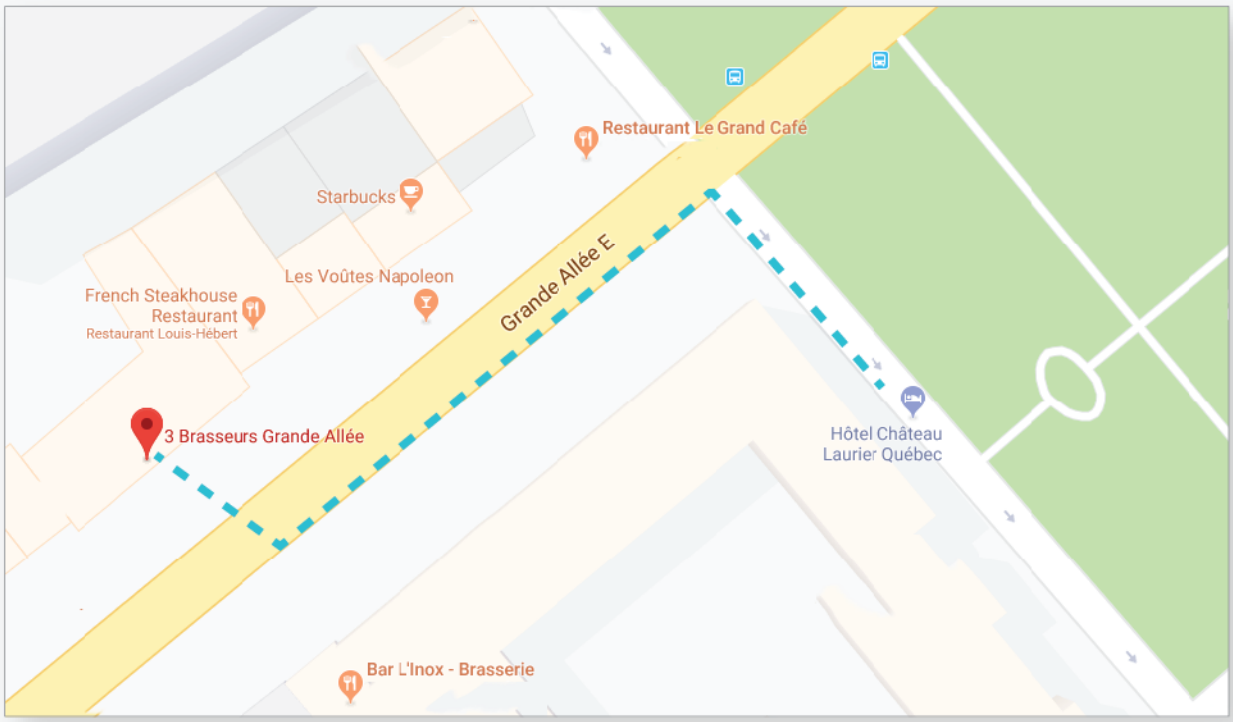
Social program

Welcoming reception and cocktail (June 2, 8:15pm)

Des Plaines Ballroom or Jardin des Quatre saisons Courtyard (weather permitting)
All registered participants and accompanying guests are invited.
Admission included in registration fees.

Pub dinner (June 4, 7:30 pm)

Optional. Admission, $40/person, paid at registration time; extra places might be available at registration desk (please enquire).
Location: Les Trois Brasseurs, 650 Grande-Allée Est
Walking directions below; about 100 m from meeting site (Château Laurier)



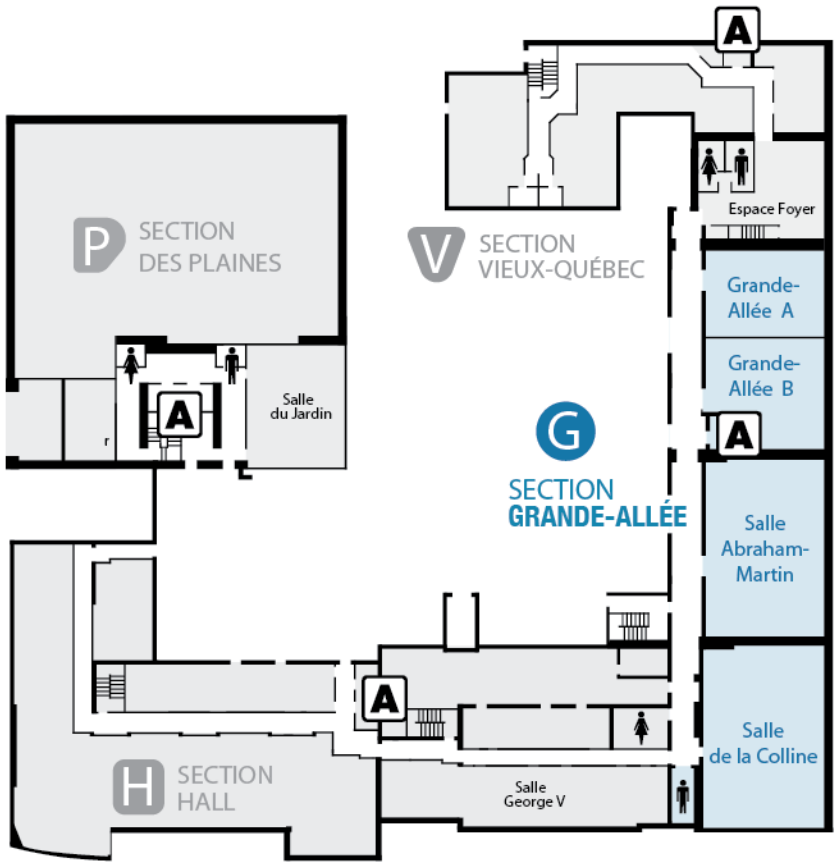
Hotel floor plan

GROUND FLOOR

Main meeting areas are indicated

1ST FLOOR

Main meeting areas are indicated



- Elevators
- Restaurant (Le St-Hubert)
- Resto-Bar
- Convenience store
- Pool and sauna
- Fitness center
- Massage room
- Locker room

Scientific Program

Neutrophil 2018 International Symposium
Hôtel Château Laurier, Québec, Qc, Canada

Saturday, June 2

15h00-18h00	On-site registration begins
19h00	**Introductory remarks** Co-chairs (P McDonald, PE Poubelle)
19h15-20h00	**Keynote Lecture: Dr Paul Kubes** (University of Calgary, Canada) *Live fast, die young, and leave a good-looking corpse*
20h15	Welcoming Reception and Cocktail (Des Plaines Ballroom or Jardin des Quatre saisons Courtyard, weather permitting)

Sunday, June 3

8h00 Continental breakfast (Des Plaines Ballroom)

Session I: Classic Neutrophil Functions

Chairs: SG Bourgoin, M Pouliot, OE Sørensen

8h30- 9h00	Ole E. Sørensen (Lunds Universitet, Lund, Sweden) *A life in science devoted to neutrophil granules (In memoriam, Dr Niels Borregaard)*
9h00- 9h30	Anthony W. Segal (University College London, London, UK) *Vacuolar pH, its regulation and role in microbial killing*
9h30- 10h00	Anna Huttenlocher (University of Wisconsin, Madison, USA) *Reverse neutrophil migration and resolution of inflammation*
10h00- 10h30	Hongbo R. Luo (Harvard Medical School, Boston, USA) *Regulation of neutrophil function by inositol hexakisphosphate kinase 1 (IP6K1)*
10h30-10h45	Coffee break
10h45-11h15	Edwin R. Chilvers (University of Cambridge, UK) *Neutrophil priming: a one way ticket?*

Selected talks from abstracts

11h15- 11h30	Jennifer C. Brazil (University of Michigan, USA) *N-linked glycans on CD11b/CD18 regulate PMN transepithelial migration*
11h30- 11h45	Sergio D. Catz (The Scripps Research Institute, USA) *JFC1 regulates Rac1-GTP uropod localization to control neutrophil directional sensing, chemotaxis and in vivo migration*

Poster session

11h45-12h30	Poster rooms
12h30-13h30	Lunch

Session II: Neutrophil Extracellular Traps

Chairs: J-P Lavoie, PP McDonald, DD Wagner

13h30- 14h00	Denisa D. Wagner (Harvard Medical School, Boston, USA) *NETs: nuclear weapons of inflammation*
14h00- 14h30	Johan van der Vlag (Radboud University, Nijmegen, The Netherlands) *The ins and outs of NETs*
14h30- 15h00	Balázs Rada (University of Georgia, Athens, GA, USA) *Neutrophil extracellular traps in cystic fibrosis airway disease*
15h00- 15h30	Martin Herrmann (Friedrich-Alexander-Universität, Erlangen, Germany) *Neutrophils, NETs and aggNETs contribute to initiation and resolution of inflammation*
15h30-15h45	Coffee break
15h45- 16h15	Discussion/workshop on NETs

Trainees' Session : Part 1

Chairs: Sarah Walmsley, Marc Pouliot

16h15- 16h27	Ania Bogoslowski (University of Calgary, Canada) *Reconnaissance neutrophils survey lymph nodes at steady state*
16h27-16h39	Laurence S.C. Lok (Cambridge University Hospitals, UK) The *Homeostatic role of lymph node-resident neutrophils in adaptive immunity*
16h39-16h51	Immanuel Kwok (Singapore Immunology Network) *Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking and effector functions*
16h51-17h03	Brian Hsu (McGill University, Montréal, Canada) *Immature neutrophils promote breast cancer liver metastasis by suppressing NK cell-mediated immunosurveillance*

Poster session

17h20- 18h20	Poster rooms

Monday, June 4

8h00 Continental breakfast (Des Plaines Ballroom)

Session III: Emerging Topics in Neutrophil Biology

Chairs: M Fernandes, T Lämmerman, S Renshaw

8h30 - 9h00 Tim Lämmerman (Max-Planck-Institut, Freiburg, Germany)
Neutrophil swarming at sites of inflammation and infection

9h00 - 9h30 Sarah Walmsley (University of Edinbugh, UK)
Hypoxia, metabolism and neutrophilic inflammation

9h30 - 10h00 Venizelos Papayanopoulos (The Francis Crick Institute, London, UK)
Neutrophils in the regulation of inflammation

Selected talks from abstracts

10h00- 10h15 Helen L. Wright (University of Liverpool, UK)
Proteomic analysis of neutrophil extracellular traps

10h15- 10h30 Mihaela Gadjeva (Harvard Medical School, Boston, USA)
Neutrophil maturation and their response to infectious pathogens are regulated by microbiota

10h30 -10h45 Coffee break

Session IV: Plenary Workshops

NOMENCLATURE OF NEUTROPHIL SUBSETS / NEW METHODOLOGIES

Chairs: M. Glogauer, J-P Lavoie, PE Poubelle

10h45 - 11h00 Michael Glogauer (University of Toronto, Canada)
A pro-inflammatory first responding neutrophil activation subset is augmented during emergency granulopoiesis and neutropenia

11h00 - 11h15 Steven W. Edwards (University of Liverpool, UK)
Genetic reprogramming in human neutrophils during inflammation

11h15 - 11h30 Leo Koenderman (University Medical Center Utrecht, The Netherlands)
Kinetics of human neutrophil subsets in blood and bone marrow

11h30 - 11h45 Patrizia Scapini (Università di Verona, Italy)
Neutrophil heterogeneity in acute and chronic inflammatory conditions

11h45 - 12h00 Daniel Irimia (Harvard Medical School, Boston, USA)
Microscale tools for probing neutrophil sepsis, swarming, and interactions with microbes

12h00 - 12h45 Discussion on Neutrophil subsets / New methodologies

12h45 - 14h00 Lunch

Trainees' Session : Part 2

Chairs: S Walmsley, M Pouliot

14h00 -14h12 Maximilian Mauler (Heart Center, University of Freiburg, Germany)
Neutrophil transmigration during myocardial reperfusion injury is boosted by platelet serotonin

Trainees' Session Part 2 (*continued*)

14h12 - 14h24 Coraline Radermecker (GIGA-R, University of Liège, Belgium)
Neutrophil extracellular traps as major determinants of asthma

14h24 - 14h36 Hannah Isles (University of Sheffield, UK)
Regulation of inflammation resolution by differential neutrophil migration patterns in zebrafish

14h36 - 14h48 Camilia Margaroli (Emory University, Atlanta, USA)
Transcriptional burst promotes functional adaptation in neutrophils recruited to cystic fibrosis airways

Home-grown science

Chairs: PP McDonald, PE Poubelle

15:00-15:30 Martin Pelletier (Université Laval, Québec, Canada)
Targeting neutrophil metabolism to control inflammatory responses

15:30-16:00 Nicolas Flamand (Université Laval, Québec, Canada)
Neutrophils and the cannabinoid system: crosstalk with eicosanoids

16h00-16h20 Coffee break

Session V: Neutrophils in Adaptive Immunity and Beyond

Chairs: M Fernandes, J Filep, MC Karlsson

16h20-16h50 Mikael C. Karlsson (Karolinska Institutet, Stockholm, Sweden)
Neutrophil regulation of autoreactive B cell responses

16h50-17h20 Luc Vallières (Université Laval, Québec, Canada)
Pervasive effects of neutrophils in B cell-dependent autoimmune demyelination

17h20-17h50 Marco A. Cassatella (Università di Verona, Verona, Italy)
Advances on the cytokine production and gene expression regulation in human neutrophils activated via TLR8

Selected talks from abstracts

17h50-18h05 Jyotika Sharma (University of North Dakota, Grand Forks, USA)
Klebsiella pneumoniae infection of neutrophils inhibits their efferocytic clearance by modulating cell death pathway

18h05-18h20 Gregory L. Kabachinski (AbbVie)
NETosis signaling is not conserved across species

18h20 Cocktails and Poster Awards (Des Plaines Ballroom)
or Jardin des Quatre saisons Courtyard (weather permitting)

19h30 Pub Dinner at *Les Trois Brasseurs* (optional)

Tuesday, June 5

8h00 Continental breakfast (Des Plaines Ballroom)

Session VI: Neutrophils in Autoimmune Disease and Cancer

Chairs: PE Poubelle, O Söhnlein

8h30- 9h00	Oliver Söhnlein (Klinikum der Universität München, Munich, Germany) *Kiss of death: neutrophils kill smooth muscle cells to induce atherosclerotic plaque destabilization*
9h00- 9h30	Mariana J. Kaplan (National Institutes of Health, Bethesda, USA) *Neutrophils and their death in the pathogenesis of systemic autoimmune diseases*
9h30- 10h00	Peter A. Nigrovic (Harvard Medical School, Boston, USA) *Modulation of neutrophil and megakaryocyte function by emperipolesis*
10h00- 10h30	Zvi Fridlender (Hadassah Medical Center, Jerusalem, Israel) *Different sub-populations of cancer-related neutrophils, their activation and recruitment into tumors*
10h30- 11h00	Stephen Renshaw (University of Sheffield, UK) *Reverse migration as a therapeutic target in inflammation resolution*

Selected talks from abstracts

11h00-11h15	Sven Brandau (University Hospital Essen, Germany) *Identity of low-density neutrophils in cancer and their relation to MDSCs*
11h15-11h30	Karin Christenson (University of Gothenburg, Sweden) *Neutrophils in inflamed joints retain phenotypic characteristics of naïve blood cells*

Concluding remarks

11h30-11h45 PP McDonald, PE Poubelle, S Renshaw

List of Abstracts (speakers)

KEYNOTE SPEAKER: **Dr. PAUL KUBES**
University of Calgary, Calgary, Canada
Live fast, die young, and leave a good-looking corpse

It has been known for many years that the neutrophil can fight infections. However, until recently, the cell has simply been thought to be recruited from the vasculature into an infected tissue where it phagocytosed bacteria and killed the pathogens via oxidants and proteases. We now know that in addition to neutrophils circulating in the blood stream, there are neutrophils that seem to be retained in tissues like lung, spleen and lymph node and that are primed to respond to bacteria in an unexpectedly rapid fashion. In many cases they phagocytose bacteria but in the blood stream they need to have help in catching these pathogens. They can do this directly by releasing NETs, or indirectly by allowing other cells to catch the bacteria and then helping to clear these pathogens. There are instances when phagocytosis is not possible and under these conditions they can release their contents in the form of NETs. By contrast in sterile injury, neutrophils also infiltrate afflicted sites but must be able to differentiate between infectious and non-infectious situations. Under sterile conditions they home into sites and can actually provide critical repair functions. Neutrophils then die, however this process remains hugely controversial and may differ between sterile and infectious conditions.

O1 Ole E. Sørensen
Lunds Universitet, Lund, Sweden
A life in science devoted to neutrophil granules
(In memoriam, Dr. Niels Borregaard)

O2 Anthony W. Segal
University College London, London, UK
Vacuolar pH, its regulation and role in microbial killing

This talk is devoted to a consideration of the way in which the NADPH oxidase of neutrophils, NOX2, functions to enable the efficient killing of bacteria and fungi. It will demonstrate that the NADPH oxidase functions to optimize the ionic and pH conditions within the vacuole for the solubilization and optimal activity of the proteins released into this compartment from the cytoplasmic granules, which kill and digest the microbes. The pH changes that occur in subsets of monocytes, macrophages and dendritic cells will also be described.

O3 Anna Huttenlocher
University of Wisconsin, Madison, USA
Reverse neutrophil migration and resolution of inflammation

Chronic inflammation leads to tissue damage and impaired wound healing. We exploit the optical transparency of zebrafish embryos to image the onset and resolution of neutrophil inflammation in real time. We will discuss the role of chemoattractant signaling in the neutrophil reverse migration and resolution of inflammation, and how this changes in the context of infection and cancer.

O4 Hongbo R. Luo
Harvard Medical School, Boston, USA
Regulation of neutrophil function by inositol hexakisphosphate kinase 1 (IP6K1)

During this talk, I will address the role of IP6K1 in controlling neutrophil function in bacterial pneumonia models. Here we report that disrupting the inositol hexakisphosphate kinase 1 (Ip6k1) gene or pharmacologically inhibiting IP6K1 activity using the specific inhibitor TNP efficiently and effectively enhanced host bacterial killing but reduced pulmonary neutrophil recruitment, minimizing the lung damage caused by both gram-positive and gram-negative bacterial pneumonia.

O5 Edwin R. Chilvers
University of Cambridge, Cambridge, UK
Neutrophil priming: a one way ticket?

The ability for priming to cause a dramatic up-regulation in the neutrophils capacity to generate superoxide anions and undergo degranulation has been long recognised. Moreover, there is a strong link between priming and the injurious potential of neutrophils.
Our group described the capacity of neutrophils to recover from the primed state in-vitro (‘de-priming’) and we have been seeking evidence of this in-vivo. Use of radiolabelled (99m-Tc) neutrophils detected using SPECT has shown that cells primed ex-vivo (PAF or GM-CSF) are trapped on first pass through the human pulmonary capillary bed but then gradually returned to the system circulation with a half-time of 40 mins, supportive of in-vivo de-priming.
To understand the mechanism of de-priming we have used single cell ‘optical stretching’ of individual neutrophils and shown that these cells are highly mechanosensitive and that repeated ‘squeezes’ causes a rapid rounding of previously primed-polarised cells. This loss of the neutrophils polarisation and restoration of normal CD11b expression was also seen when neutrophils were passed through a series of artificial 5um constrictions, designed to model the tight microvasculature constrictions of the pulmonary capillary bed. Finally, use of real time-deformability cytometry has revealed that de-priming is associated with significant increases in neutrophil area and increased softness and deformability. These data support the concept of passive and active (mechanical) de-priming and the hypothesis that the lung may serve a (physiological) role to trap primed neutrophils and actively return them to the circulation in an un-primed state.

O6 Jennifer C. Brazil

University of Michigan, Ann Arbor, USA

N-linked glycans on CD11b/CD18 regulate PMN transepithelial migration

Pathogen-triggered neutrophil (PMN) recruitment is critical for innate immunity, but aberrant PMN influx and associated mucosal tissue damage is implicated in the pathogenesis of numerous human inflammatory diseases. The critical final step in PMN trafficking into mucosal lined organs (including the gut and lungs) involves transepithelial migration (TEpM). The glycoprotein CD11b/CD18 is the predominant B2 integrin mediating human PMN TEpM. We have recently shown that targeting of the N-linked glycan Lewis X (Lex) on CD11b blocks PMN TEpM to the same extent as I domain inhibitory anti-CD11b mAbs. Given the importance of glycosylation for modulation of CD11b/CD18 function we performed tandem Mass Spec/Mass Spec analyses to generate, for the first time, a complete profile of the N-linked glycans on human CD11b/CD18. These data revealed a startling lack of sialylation and an overabundance of uncommon high mannose glycans on CD11b/CD18. Interestingly the profile of CD11b/CD18 glycans was distinct from the total profile of N-linked glycans for human PMN suggesting CD11b/CD18 specific glycans. Specific glycan epitopes identified on CD11b/CD18 included a high Mannose oligosaccharide recognized by the Galanthus Nivalis (GN) lectin and a bisected GlcNAc glycan recognized by the Phaseolus Vulgaris erythroagglutinin (EPHA) lectin. Further, we show that targeting of CD11b/CD18 with EPHA but not GN significantly inhibits PMN chemotaxis and TEpM. Given the unique glycan profile and the specific effects of CD11b/CD18 glycan targeting on PMN trafficking, these data demonstrate that specific glycans on CD11b/CD18 represent novel targets for reduction of PMN associated tissue damage in inflammatory diseases including chronic obstructive pulmonary disease and inflammatory bowel disease.

O7 Sergio D. Catz

The Scripps Research Institute, La Jolla, USA

JFC1 regulates RAC1-GTP uropod localization to control neutrophil directional sensing, chemotaxis and in vivo migration

In neutrophil chemotaxis, Rac1 has discrete functions at both the leading edge and the uropod. Rac1 GEFs are proposed to localize towards the cell front, but how Rac1 is regulated at the uropod is unknown. Using chemotaxis, exocytosis and signaling assays, vesicular trafficking and super-resolution microscopy analysis of GTP-Rac1 distribution, and neutrophil migration in chimeric mice, we show that the discrete distribution of Rac1 at the uropod is regulated by vesicular trafficking mediated by the effector JFC1, regulating neutrophil directional migration in vitro and in vivo. JFC1-null neutrophils showed defective polarization and impaired directionality to chemoattractants in vitro, while chemoattractant-induced actin remodeling and calcium signaling were normal in JFC1-/- cells. The defects were not explained by impaired exocytosis associated with JFC1-deficiency. Instead, JFC1-null cells show Rac1-GTP accumulation at the uropod and impaired tail detachment, indicating that defective Rac1-GTP recycling from the uropod causes impaired directionality. Quantitative super-resolution microscopy shows that the adjacent distribution of JFC1 and Rac1-GTP increases upon stimulation. JFC1 directly interacts with Rac1-GTP and regulates Rac1-GTP trafficking. Neutrophil dynamics in bone marrow-chimeric mice show that JFC1-/- cells are unable to move directionally towards the chemoattractant thus recapitulating in vivo the in vitro defective phenotype. Our data highlight JFC1-mediated Rac1 trafficking as a potential target to regulate neutrophil recruitment in inflammation and immunity.

O8 Denisa D. Wagner

Harvard Medical School, Boston, USA

NETs: nuclear weapons of inflammation.

Neutrophils are the first cell type to respond at inflammation sites produced by infection or injury. A newly recognized arsenal of neutrophils is their “nuclear weapon”: Neutrophil extracellular traps (NETs). How neutrophils release their chromatin to form NETs is not clear. Fuchs et al. (2007) observed the internal membranes of the cells dissolving, which was followed by cell lysis and decondensed chromatin releasing in the environment. More recently, during an infectious process, Yipp et al. (2012) observed direct secretion of nuclear contents from the cell without killing the neutrophils. The cell biology of NETosis is still a mystery; except for the role of the enzyme PAD4, which is responsible for the chromatin decondensation by arginine citrullination. Although released NETs trap microbes, they also cause damage that is proinflammatory, and the NETs themselves are highly prothrombotic.

NET formation is induced by many stimuli such as microbes, cytokines, antibodies to neutrophils, hypoxia, small crystals, and others. Ischemia, as we’ve seen in the myocardial infarction mouse model, induces NETosis. We have found that mice lacking NETosis (PAD4-/-), or mice treated with DNase 1 (an enzyme that digests DNA, the backbone of the NETs) develop smaller infarcts and their heart function is better after the ischemic event. Some conditions, such as diabetes, cancer, or old age, prime neutrophils towards NETosis. These conditions may produce excessive thrombosis and promote inflammation. NET deposition also leads to fibrosis. We observed that the spontaneous fibrosis of organs produced by aging is greatly reduced in PAD4-deficient mice. Their hearts remain as young hearts, while both the systolic and diastolic function of wild-type mice declines with age. We propose that PAD4 or NETs will be an interesting drug target in cardiovascular diseases.

O9 Johan van der Vlag
Radboud University, Nijmegen, The Netherlands
The ins and outs of NETs.

This presentation will discuss the chromatin content of NETs, and a novel method that is able to distinguish between NETs that are formed by, respectively, NADPH oxidase (NOX)-dependent and NOX-independent pathways. It appears that NETs are enriched in specific chromatin modifications that we have previously associated with apoptosis and lupus nephritis. Neutrophil elastase cleaves the N-terminal tails of core histones during NOX-dependent, but not during NOX-independent NET formation. Consequently, the detection of myeloperoxidas - histone complexes with our panel of antibodies against N-terminal histone tails allows the discrimination between NETs formed through a NOX-dependent or NOX-independent manner. Characterization of in vivo circulating NETs revealed the presence of NOX-independent NETs in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and sepsis, but NOX-dependent NETs in psoriatic arthritis (PsA). The capacity of NETs to activate endothelial cells was limited to NETs generated via NOX-independent pathways. It can be concluded that there is heterogeneity in NET-forming pathways in vivo in different disease conditions, and effector functions of NETs formed by different pathways, which could lead to disease-specific strategies to prevent NET-mediated pathologies.

O10 Balázs Rada
University of Georgia, Athens, USA
Neutrophil extracellular traps in cystic fibrosis airway disease.

Cystic fibrosis (CF) airway disease is characterized by chronic presence of neutrophil granulocytes. Although formation of neutrophil extracellular traps (NETs), an antimicrobial trapping mechanism of neutrophils, occurs in CF airways, it remains unclear whether NETs are advantageous or disadvantageous for CF patients. NETs could fight respiratory pathogens but could also deliver lung-damaging neutrophil cargo to the airway lumen. During this talk, I will address 1) the mechanism of NET formation stimulated by Pseudomonas aeruginosa, one of the main respiratory pathogens in CF, 2) the effect of NETs on P. aeruginosa behavior, 3) NET formation in CF neutrophils and 4) links between NET-specific markers and CF lung disease.

O11 Martin Herrmann
Friedrich-Alexander-Universität, Erlangen, Germany
Neutrophils, NETs and aggNETs contribute to initiation and resolution of inflammation.

NET formation the regulated externalization of neutrophil-borne chromatin is a process involved in the first line defense against invading pathogens. In vivo NET formation mostly takes place in cavities, ducts, or lacunae of a plethora of tissues. During NET formations at low neutrophil densities pro-inflammatory mediators are instantly released and substantially contribute to inflammation, tissue destruction, and to the recruitment of further leukocytes to the site of inflammation. Consequently this leads to a huge number of neutrophils that release their chromatin and form further NETs that tend to aggregate and form aggregated NETs (aggNETs). These are cellular units that contain several types of viable cells, dead cell detritus, microorganisms, and waste bound by NETs. The aggNETs are endowed with robust proteolytic activities that digest the cytokine load sequestered by the DNA scaffold. This orchestrates the resolution of inflammation at high neutrophil densities and initiates the post-inflammatory healing processes. However, if the aggNETs occlude the ducts they are surveying a secondary inflammatory pathology may emerge.

O12 Ania Bogoslowski
University of Calgary, Calgary, Canada
Reconnaissance neutrophils survey lymph nodes at steady state

There is growing evidence that the lymph node may function not only as an adaptive immune organ but also as part of the innate defence system against pathogens. We have discovered a previously undescribed population of neutrophils residing in the lymph node at steady state. Using advanced intravital imaging of the lymph node, we see that neutrophils in close proximity to lymph node blood vessels are fairly stationary while neutrophils in the subcapsular sinus are more patrolling in nature. L-selectin is necessary for neutrophils to accumulate in the lymph node at steady state, but is independent of P-Selectin glycoprotein ligand -1, which is critical for neutrophil recruitment in the periphery. Neutrophils seem to turnover completely within 24h and annexin V expression is not increased on these neutrophils, suggesting that neutrophils are not dying in the lymph node. In addition, 48hr treatment with FTY720, an inhibitor of Sphingosine-phosphate receptor 1(S1P1), results in a significant increase in neutrophil numbers in the lymph node, suggesting the S1P1 is necessary for neutrophil egress from the lymph node. These neutrophils may contribute as a first level of defence in the lymph node, which then recruit additional neutrophils through a signal amplification system. 48h treatment with anti-L-selectin was exploited to reduce steady state neutrophil numbers in the lymph node. After this treatment, mice were infected in the footpad and neutrophil recruitment to the lymph node (but not infection site) was found to be reduced at 1h, 2h, and 4h post infection. By 8h, neutrophil numbers are restored to control levels, emphasizing the early role of steady state neutrophils. This work is critical to our understanding of how the lymph node functions as an immune barrier for pathogens.

O13 Laurence S.C. Lok
Cambridge University Hospitals, Cambridge, UK
The homeostatic role of lymph node-resident neutrophils in adaptive immunity

Introduction: Neutrophils play a key role in innate immunity, but can also shape the adaptive immune response. Whilst neutrophils can migrate to draining lymph nodes (LNs) following infectious challenges, the role of tissue-resident neutrophils in physiological settings is unclear. We hypothesise that neutrophils homeostatically traffick into and are resident within LNs, and can influence adaptive immunity.

Methods: LNs from unchallenged C57BL/6 or LysM-GFP mice and from human organ donors were harvested for flow cytometry and confocal microscopy. Neutrophil dynamic behaviour in vivo was examined by intravital imaging of popliteal LNs. Isolated neutrophils were stimulated with immune complex (IC) ex vivo and co-cultured with T cells.

Results: Neutrophils were present in LNs from unchallenged mice; the majority (75-82%) were located in LN tissues, in interfollicular and interlobar areas. Intravenously transferred neutrophils were present in LNs up to 7 days post transfer. Intravital imaging showed that transferred neutrophils trafficked into LNs via blood and lymphatic vessels, and their recruitment was impaired by peripheral lymph node addresin (PNAd) blockade (mean neutrophils per field of view, 13.2 control vs 4.8 anti-PNAd, $p < 0.01$). LN neutrophils showed higher surface major histocompatibility complex II (MHCII) expression compared to blood neutrophils. Neutrophils were also present in human LNs, and expressed surface MHCII. Upon ex vivo IC stimulation, neutrophils upregulated expressions of MHCII, CD40 and CD86, and increased T cell activation. In vivo, neutrophils were capable of the uptake into LNs of systemically administered IC.

Conclusion: Neutrophils are resident within LNs under homeostatic conditions, and are capable of expressing MHCII, thereby influencing adaptive immunity.

O14 Immanuel Kwok
Singapore Immunology Network, Singapore
Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking and effector functions

Neutrophils are specialized cells in the early innate immune response, requiring constant replenishment from proliferative bone marrow (BM) precursors because of their short half-lives. While it is well established that neutrophils are derived from the granulocyte-macrophage progenitor (GMP), the differentiation pathways from GMP to functional mature neutrophils are poorly defined. Using mass cytometry (cyTOF) and cell cycle-based analysis, we identified three neutrophil subsets within the BM: (1) a committed neutrophil precursor (preNeu) positioned in proliferative clusters that sequentially differentiates into (2) non-proliferating immature neutrophils and (3) mature neutrophils. Transcriptomic profiling and functional analysis revealed that the proliferative program of preNeu is substituted by the gain of migratory and effector functions as cells mature. Consequently, preNeus expand upon inflammatory and tumoral stress, while immature neutrophils are recruited to the periphery of tumor-bearing mice. In summary, our study identifies specialized granulocytic populations in the BM that ensure supply under homeostasis and stress responses.

O15 Brian Hsu
McGill University, Montréal, Canada
Immature neutrophils promote breast cancer liver metastasis by suppressing NK cell-mediated immunosurveillance

Liver represents a prominent site for breast cancer metastasis, with 50-70% of women with metastatic breast cancer developing hepatic metastases. Immune cells can either impair the metastatic process or conversely, assist in the seeding, colonization, and growth of disseminated cancer cells. Neutrophils, which have originally known to eliminate pathogens by a variety of mechanisms including phagocytosis, cytokine release, reactive oxygen species production and the release of neutrophil extracellular traps, have recently known to play a key role in the control of tumor progression. Neutrophils are phenotypically heterogeneous and, depending on the context, exert anti- and pro-metastatic functions. Here, we demonstrate that tumor cells capable of forming liver metastases induce the accumulation of neutrophils in the liver parenchyma and peripheral blood. Cancer cell-derived G-CSF, in concert with TGF-beta1, mobilize immature neutrophils that promote liver metastasis. Our data also indicate that these immature neutrophils do not impair T cell proliferation/activation; but rather, suppress the ability of NK cells to kill tumor cells. NK cells infiltrating growing liver metastases switch expression from activating to inhibitory NK receptors, which corresponds with an influx of immature neutrophils expressing an inhibitory NK ligand (CD155). Altogether, these data unravel a novel mechanism by which neutrophils can facilitate tumor progression leading to liver metastasis.

O16 Tim Lämmerman
Max-Planck-Institut, Freiburg, Germany
Neutrophil swarming at sites of inflammation and infection

Recent intravital microscopy studies of neutrophil populations directly at the site of tissue damage or microbial invasion have changed our perspective on neutrophil responses within tissues. Swarm-like migration patterns of neutrophils, referred to as 'neutrophil swarming', have been detected in diverse tissues under conditions of sterile inflammation and infection with various pathogens, including bacteria, fungi, and parasites. Current work has begun to unravel the molecular pathways choreographing the sequential phases of highly coordinated chemotaxis followed by neutrophil accumulation and the formation of substantial neutrophil clusters. This talk will address how intercellular communication among neutrophils provides them with a level of self-organization during neutrophil swarming.

O17 Sarah Walmsley
University of Edinburgh, Edinburgh, UK
Hypoxia, metabolism and neutrophilic inflammation

Neutrophils are essential for bacterial clearance and unique in their ability to survive and function in tissues where oxygen and nutrients are limited. In this talk I will explore how the functional adaptation of inflammatory neutrophils is a consequence of the interplay between oxygen and metabolite sensing pathways. These adaptive responses are also important in disease pathophysiology, where exaggerated metabolic activation of neutrophils is associated with a disproportionate inflammatory response that is detrimental to the host. The importance of neutrophil mediated morbidity and mortality will be discussed in the context of concurrent infection and systemic hypoxia with early mechanistic insights explored around the central role of the hypoxia inducible transcription factor HIF-1alpha.

This work was principally supported by a Wellcome Trust Senior Clinical Fellowship award

O18 Venizelos Papayanopoulos
The Francis Crick Institute, London, UK
Neutrophils in the regulation of inflammation

Neutrophils are essential phagocytes of the innate immune system. They engage pathogens via degranulation, phagocytosis and the release of extracellular web-like structures called neutrophil extracellular traps (NETs). Recent findings highlight novel immunomodulatory roles for these cells, particularly in the regulation of inflammation. Under sterile conditions, NET release promotes inflammation and atherosclerosis by turning on pro-inflammatory cytokine expression in lesion-associated macrophages. Moreover, during infection neutrophils regulate their own recruitment conditionally by adjusting their cytokine expression according to the size of the microbes they encounter. The differential localization of reactive oxygen species (ROS) generated in response to microbes of different size plays a critical role in regulating interleukin-1β expression and the formation of neutrophil clusters required to clear large microbes.

O19 Helen L. Wright
University of Liverpool, Liverpool, UK
Proteomic analysis of neutrophil extracellular traps

Neutrophil extracellular traps (NETs) may be implicated in the development of auto-immunity via externalisation of intracellular auto-antigens. The aim of this work was to use quantitative proteomics to identify and measure NET proteins.

Ultra-pure neutrophils from healthy individuals (n=3), patients with rheumatoid arthritis (RA, n=6) and patients with lupus (SLE, n=6) were incubated ±PMA (50nM, PKC super-activator) or A23187 calcium ionophore (3.8μM, activator of protein-arginase deiminase-4 (PAD4)) for 4h. DNA was digested. NET proteins were concentrated onto Strataclean beads and digested on-bead with trypsin. Peptides were resolved on 1h gradients. Data-dependent LC-MSMS analyses were conducted on a QExactive HF quadrupole-Orbitrap mass spectrometer. Label-free protein quantification was carried out using Progenesis QI. Immunofluorescent staining was used to confirm proteomics data.

97 proteins were differentially expressed on PMA-NETs and A23187-NETs from healthy controls (p2, peptides >2), including histones H3.1 and H2A, cathepsin G and neutrophil elastase (higher in PMA-NETs), and histones H1.0, H1.5 and H1.4 and PAD4 (higher in A23187-NETs). Similar expression of histones and granule proteins was observed on RA and SLE NETs in response to PMA and A23187. However, cathelicidin (LL37), CRISP3, lipocalin and MMP8 were significantly higher on RA A23187-NETs, whereas histones H1.0 and H2B were higher on SLE A23187-NETs. Leukocyte elastase inhibitor SERPIN1B and thymidine phosphorylase were higher on SLE PMA-NETs. Several proteins contained citrulline modifications including histones and granule enzymes.

This work demonstrates that NET protein antigens are disease and stimulus specific and provides insight into the molecular pathology of RA and SLE.

O20 Mihaela Gadjeva
Brigham & Women's Hospital, Harvard Medical School, USA
Neutrophil maturation and their response to infectious pathogens are regulated by microbiota

It has long been considered that a neutrophil's response to various infectious challenges is innately pre-determined. Here, we provide data that demonstrates that neutrophil transcriptomes and proteomes are modulated by the microbiota. Using RNAseq and quantitative proteomic approaches we defined mature neutrophil transcriptome and proteome signatures at steady state and during ocular infection with Pseudomonas aeruginosa. We found that the proteomic signatures of mature neutrophils derived from the GF and SPF were significantly different. GF-serum exposed neutrophil progenitors did not mature efficiently and had compromised bactericidal properties when compared to progenitors matured in SPF-derived serum. To identify molecular pathways, we set-up an in vitro system where neutrophil progenitors were transduced with lentiguides to knock-down key microbiota-driven pathway gene targets. We are currently examining the maturation and bactericidal characteristics of "CRISPR-ed" neutrophils.

During infection with P. aeruginosa, GF-derived neutrophils responded with alterations in transcriptional regulation, ncRNA processing, rRNA metabolic processes, mRNA processing, and splicing, while the SPF-derived neutrophils responded with alterations in acetylation. Consistently, GF mice were more susceptible to ocular keratitis compared to SPF controls.

O21 Michael Glogauer
University of Toronto, Toronto, Canada
A pro-inflammatory first responding neutrophil activation subset is augmented during emergency granulopoiesis and neutropenia

Neutrophils are considered a homogenous circulating leukocyte population with the exception of specific subsets identified in disease. Little is known about the possible activation states of these cells in health and during acute inflammation. Through cluster of differentiation (CD) marker analysis, we identified a pro-inflammatory neutrophil (piN) subpopulation in healthy mouse and human, which have elevated surface expression of activation markers compared to the bulk resting state neutrophils (rsNs). In response to acute tissue inflammation, piNs are rapidly depleted from the circulation coincident with early tissue recruitment, consistent with a first responder phenotype. At later stages of acute peritoneal inflammation piNs became the dominant neutrophil population in bone marrow and in blood, gradually returning to baseline levels with resolution of inflammation. High levels of piNs were observed in neutropenic mice and pediatric neutropenic patients who were resistant to infection, highlighting an important role of piNs in immunosurveillance.

O22 Steven W. Edwards
University of Liverpool, Liverpool, UK
Genetic reprogramming in human neutrophils during inflammation

It is now recognised that human neutrophils have considerable functional plasticity and altered functions in different scenarios of inflammation or infection. In some instances, this functional heterogeneity arises from molecular re-arrangements, such as changes in the surface expression of plasma membrane receptors, but in other cases this arises as a consequence of differential changes in gene expression. This talk will describe the changes in neutrophil gene expression that are observed in inflammatory conditions or infections and how this may predict either the function of these activated cells, or help identify the agonists that regulate their function in disease.

O23 Leo Koenderman
University Medical Center Utrecht, The Netherlands
Kinetics of human neutrophil subsets in blood and bone marrow

During this talk, I will address the issue whether immune suppressive neutrophils (CD62Ldim) are originating from a separate pool of cells in the bone marrow. Controlled acute inflammation evoked by experimental endotoxemia is associated with a fast mobilization of these immune suppressive cells. These cells are characterized by a low potency to contain phagocytosed bacteria making them hiding places for these pathogens. In addition, these cells are characterized by a specific proteome that does not imply short-term activation. Experiments with bone marrow of normal volunteers indicate that a pool of these cells already reside in the bone marrow during homeostasis. These data imply that suppressive neutrophils are produced by a parallel differentiation pathway in the human bone marrow.

O24 Patrizia Scapini
Università di Verona, Verona, Italy
Neutrophil heterogeneity in acute and chronic inflammatory conditions

Recent findings have uncovered novel fascinating aspects of the biology of neutrophils, which ultimately attribute to these cells a broader role in inflammation and immunity. One aspect that is currently under intensive investigation is the notion of neutrophil "heterogeneity". Studies examining neutrophils in a variety of acute and chronic inflammatory conditions report, in fact, the recovery of CD66b+ cells displaying neutrophil-like morphology and able to exert either immunosuppressive or proinflammatory properties. Some of these neutrophil populations sediment within the peripheral blood mononuclear cell (PBMC) fraction after density gradient centrifugation of blood, and are thus generally defined as "low density neutrophils" (LDNs). The various LDN populations to date identified and described in pathological settings are heterogeneously composed by mixed populations of activated mature neutrophils, as well as immature neutrophils at different stages of differentiation. In this context, in a recent study, we demonstrated that in healthy volunteers receiving G-CSF for stem cell mobilization (GDs) mature CD66b+CD10+ LDNs, as well as CD66b+CD10+ normal density neutrophil (NDNs), inhibit proliferation and IFNγ production by T cells via a CD18-mediated contact-dependent release of arginase 1(ARG1). By contrast, immature CD66b+CD10- LDNs from GDs manifest an opposite behaviour, since they promoted T cell survival and enhanced proliferation and IFNγ production by T cells via CD18-mediated contact-dependent mechanisms. Our findings suggest that it is becoming mandatory to develop new experimental approaches that may allow a precise separation and careful characterization of each mature and immature neutrophil populations appearing into the circulation during a given disease, in terms of phenotype, transcriptional profile and functions.

O25 Daniel Irimia
Harvard Medical School, Boston, USA
Microscale tools for probing neutrophil sepsis, swarming, and interactions with microbes

I will discuss three emerging microfluidic technologies for probing neutrophil functions, which enable unexpected insights into the biology of neutrophil swarming, netosis after neutrophil-pathogen interactions, and the diagnosis of sepsis in one drop of blood.

O26 Maximilian Mauler

Heart Center, University of Freiburg, Germany

Neutrophil transmigration during myocardial reperfusion injury is boosted by platelet serotonin

Neutrophils are the dominant immune cell that infiltrates the heart during the inflammatory response of reperfusion after myocardial infarction (MI). Acute neutrophil recruitment and transmigration is partly triggered by platelet serotonin (5-HT). We aimed to evaluate how 5-HT drives neutrophil response during myocardial reperfusion injury.

MI was induced in wild type (WT) and mice deficient for tryptophan hydroxylase 1 (TPH1-/-; rate limiting enzyme for 5-HT synthesis) followed by reperfusion. Heart function and infarct size were evaluated. Heart tissue and blood was screened for inflammatory cytokines and adhesion molecules.

Serotonin peaked 24h after MI in WT mice (150 ng/mL) whereas Tph1-/- mice had no detectable 5-HT levels in plasma. This led to improved cardiac function and reduced infarct size in Tph1-/- mice (35 vs 54 in WT; % area at risk (AAR)). Tph1-/- mice had 50% less neutrophils within the AAR (14 vs 28 in WT per mm2 tissue) and reduced MPO levels in heart and plasma while monocyte and macrophage distribution was similar to WT. Circulating neutrophils had 50% reduced CD11b expression in Tph1-/- mice whereas CD11a, CD18, and PSGL-1 was not affected. In vitro stimulation of neutrophils with 5-HT revealed fewer granules within the cell (electron microscopy), increased CD11b on the cell surface (FACS), and elevated MPO levels in the supernatant (ELISA).

Platelet serotonin orchestrates neutrophil migration during myocardial reperfusion injury by inducing degranulation and subsequent upregulation of CD11b and release of MPO. This accelerates tissue damage and we propose intervening in 5-HT triggered neutrophil response could provide novel anti-thromboinflammatory treatment options.

O27 Coraline Radermecker

GIGA-R, University of Liège, Liège, Belgium

Neutrophil extracellular traps as major determinants of asthma

Environmental changes are responsible for the dramatic rise in the prevalence of allergic asthma worldwide. Decreased exposure to microbial products such as lipopolysaccharide (LPS) and respiratory viral infections represent two major risk factors for asthma, yet the mechanisms linking such conditions and host allergic susceptibility remain unclear. First, we developed two mouse models, a virus-induced asthma model and a model of asthma promoted by exposition to low LPS doses. In these models, only previously infected mice or mice exposed to low LPS doses displayed the characteristics of asthma following sensitization and challenge to house dust mite (HDM). Then, using single-cell RNA sequencing, we found that pro-allergic environments (low LPS doses and respiratory virus) induced the recruitment into the lungs of a same CXCR4hiCD49dhighLAMP-1high neutrophil subset releasing neutrophil extracellular traps (NETs). The role of NETs in asthma onset was then demonstrated using three NETosis inhibitors in our two models. Infected or low LPS doses exposed mice exhibited strong decrease of all asthma features when treated with NETs inhibitors compared to non-treated mice. Finally, to address how NETs promote the development of a Th2 immune response, we analysed by flow cytometry the distinct subpopulations of lung dendritic cells (DCs) in our models. We observed, during the NETs release phase, a recruitment of monocytic-derived DCs responsible for allergic sensitization. This recruitment was abrogated when NETS were inhibited. In conclusion, our study reveals how apparently unrelated environmental risk factors commonly shape immune responses, by recruiting a same particular subpopulation of neutrophils which release NETs, to promote asthma.

O28 Hannah Isles

University of Sheffield, Sheffield, UK

Regulation of inflammation resolution by differential neutrophil migration patterns in zebrafish

There currently exists no cure for respiratory inflammatory diseases including COPD, ARDS and cystic fibrosis. The excessive tissue damage characteristic of these diseases is caused by the inappropriate retention of neutrophils in the lungs. Understanding the molecular controllers of neutrophil migration during inflammation is crucial to develop new therapies to treat these diseases. The identification of novel neutrophil migration patterns including neutrophil swarming and reverse migration add complexity to the modulation of neutrophil migration and retention within inflamed tissue, the precise molecular mechanisms of which remain to be fully elucidated.

Here we use a zebrafish model of spontaneously resolving inflammation to study the effect of differential neutrophil migration on the outcome of inflammation. This study aims to investigate the role of the CXCR4/CXCL12 signalling axis in modulating inflammation resolution, looking specifically at neutrophil swarm resolution and reverse migration.

Inflammation is induced in larvae by tail fin transection. Assessment of neutrophil numbers at the wound site in Tg(mpx:GFP) larvae identified that pharmacological inhibition of CXCR4 accelerates inflammation resolution. Reverse migration assays using Tg(mpx:kaede) larvae show that CXCR4 inhibition accelerates neutrophil reverse migration. Increased numbers of neutrophils are recruited to wound sites in larvae where neutrophil swarming is observed, and a subsequent delay in inflammation resolution is observed in these larvae. The findings of this study suggest that CXCR4/CXCL12 signalling plays a role in modulating the resolution of inflammation. Ongoing work to develop CRISPR interference to inhibit signalling through CXCR4/CXCL12 specifically in neutrophils is promising for future.

O29 Camilia Margaroli
Emory University, Atlanta, USA
Transcriptional burst promotes functional adaptation in neutrophils recruited to cystic fibrosis airways

Rationale: Polymorphonuclear neutrophils (PMNs) are generally viewed as pre-programmed, with a short lifespan and little opportunity to deviate from their typical fates. In cystic fibrosis (CF) airway disease, PMNs display complex changes consistent with a distinct, acquired fate. Here we investigated whether this new fate was linked to de novo transcription.

Results: PMNs migrated to CF airway fluid in vitro (Forrest et al., J Leukoc Biol, 2018), and PMNs collected from CF airways in vivo increased their RNA content more than 5-fold compared to matched blood PMNs. Significantly different genes and pathways observed were consistent with: (a) increased survival and reduced apoptosis, (b) changes in immunological signatures from pro-inflammatory to regulatory, (c) absence of primary granule protein transcripts. Proteomics analysis of in vitro migrated PMNs validated the observed RNASeq profile, supporting the notion that transcriptional burst enables PMNs metabolic licensing to the CF airway microenvironment and downstream functional adaptation, including a refractory state to bacterial killing.

Conclusions: These results suggest that PMNs survive and adapt in the hostile CF airway environment via de novo transcription. However, this does not include RNAs for genes expressed in early developmental stages, for which a transcriptional block may exist. These findings challenge the conventional paradigm that holds PMNs as short-lived and transcriptionally silent cells, and open exciting avenues for PMN-targeted therapies in CF.

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O30 Martin Pelletier
Université Laval, Québec, Canada
Targeting neutrophil metabolism to control inflammatory responses

Energy metabolism ensures proper function of the immune system, as immune cells require energy not only for housekeeping functions, but also to mount specific immune responses. The regulation of immune cells' metabolism has become a major area of investigation in basic immunology, and a renewed interest in targeting metabolic is now an avenue to propose and study new immunotherapies. Among immune cells, neutrophils present a metabolic particularity by being highly glycolytic cells, in part due to their low mitochondria count and their use of oxygen to perform anti-microbial functions. I will address here different findings that my research group investigated in neutrophils and will present promising strategies to target neutrophil metabolism in order to control their cellular responses to pro-inflammatory cytokines.

O31 Nicolas Flamand
Université Laval, Québec, Canada
Neutrophils and the cannabinoid system: crosstalk with eicosanoids

Human neutrophils are recognized as an important source of bioactive lipids derived from arachidonic acid, notably leukotriene LT B_4 and prostaglandin PG E_2. They are also involved at regulating the tone of endocannabinoids, which are potent anti-inflammatory lipids. Indeed, we previously showed that human neutrophils were expert at inactivating arachidonic acid-containing endocannabinoids. During this talk, I will discuss the roles of endocannabinoids in the regulation of inflammation and how neutrophils participate to the synthesis and the degradation of endocannabinoids, as well as the strategies that could be utilized to enhance endocannabinoid levels in the hope of diminishing the inflammatory cascade involving neutrophils.

O32 Mikael C Karlsson
Karolinska Institutet, Stockholm, Sweden
Neutrophil regulation of autoreactive B cell responses.

In my talk, I will discuss our findings on how Neutrophils interact with Invariant natural killer T cells (iNKT) cells to regulate B cell responses. iNKT serve as early rapid responders in the innate recognition of both self and foreign antigens and can both enhance and negatively regulate B cell activation. The negative regulation has been shown to be induced during auto-inflammation in response to the innate cytokine IL-18. Mechanistically, IL-18 activated iNKT cells regulate self-reactive B cell activation by preventing B cell entry into the germinal center. We have recently shown that this pathway involves neutrophils that license the iNKT cells to regulate B cells through FAS ligand mediated killing. Since activation of iNKT cells using exogenous glycolipid agonists has shown great clinical potential, we investigated whether iNKT cells exert their regulatory function and how iNKT cells respond to exogenous glycolipids during inflammation in response to IL-18. We find that exogenous glycolipids disrupt the regulatory cellular cooperation between neutrophils and iNKT cells and that iNKT cells get activated to become iNKT follicular helper cells. This in effect enhances the autoreactive B cell response including production of auto-antibodies. These findings have relevance for autoimmune diseases where infections and vaccination using glycolipids that provide ligands for iNKT cells could disrupt a potentially important negative regulatory loop and allow for autoreactive B cells to enter the germinal center.

O33 Luc Vallières
Université Laval, Québec, Canada
Pervasive effects of neutrophils in B cell-dependent autoimmune demyelination

Neutrophils contribute to neuroinflammatory diseases such as neuromyelitis optica, some forms of multiple sclerosis, and the animal model experimental autoimmune encephalomyelitis (EAE). Yet despite growing interest, there has been a glaring lack of investigation into the properties of CNS-infiltrating neutrophils and their relevance to disease progression to date. In this presentation, I will demonstrate that CNS-infiltrating ICAM1+ neutrophils exhibit a remarkable plasticity during EAE: through transcriptional remodeling, they acquire macrophage-like properties in situ, including the potential for immunostimulation and MHCII-mediated antigen presentation. I will also show, in a new B-cell-dependent EAE model, that neutrophils can use the retroviral-like protease ASPRV1 to promote the chronic phase of EAE, a unique function neutrophils do not share with macrophages or other immune cells. Therefore, neutrophil-related proteins such as ASPRV1 may serve as biomarkers and therapeutic targets for demyelinating autoimmune diseases.

O34 Marco A. Cassatella
Università di Verona, Verona, Italy
Advances on the cytokine production and of gene expression regulation in human neutrophils activated via TLR8

Data published in the last decades have highlighted that neutrophils, in addition to their crucial role in pathogen killing, may also contribute to the development of innate and adaptive immunity by performing previously unanticipated functions, including the capacity to extend their survival, the competence to directly interact with other leukocytes or cell types, and the ability to produce and release a variety of cytokines. In this context, my lab has recently identified TLR8 ligands as very potent agonists activating cytokine gene expression and production by human neutrophils, likely for their ability to modify the chromatin. It is worth recalling here that TLR8 has been implicated in the sensing of viruses and more recently also bacteria, and that many TLR8 ligands have been confirmed as promising adjuvant candidates. During my talk, I will present recent findings obtained from experiments aimed at extending our knowledge on the global modulation of gene expression in human neutrophils treated with TLR8 agonists, as well as on the identification of its molecular mechanisms/players.

O35 Jyotika Sharma
University of North Dakota, Grand Forks, USA
Klebsiella pneumoniae infection of neutrophils inhibits their efferocytic clearance by modulating cell death pathway

Neutrophils are the first infiltrating cell type required for combating pneumoseptic infections by bacterial pathogens including Klebsiella pneumoniae (KPn). Clearance of infiltrating cells via a highly regulated process called efferocytosis is required for restoration of homeostasis following an infection or injury, but little is known regarding the effect of bacterial infection on this process. Here we demonstrate that KPn infection inhibits the efferocytic uptake of neutrophils in-vitro and in-vivo in lungs by macrophages. This impaired efferocytosis of infected neutrophils coincides with drastic reduction in the neutrophil surface exposure of apoptosis signature phospholipid phosphatidyserine (PS). Concomitantly, pharmacological enhancement of PS externalization restored the efferocytosis of KPn infected neutrophils. We further show that KPn infection inhibits apoptosis and instead activates non-apoptotic programmed cell death via necroptosis pathway in neutrophils. Accordingly, pharmacological inhibition of necroptosis by RIPK1 and RIPK3 inhibitors restored the efferocytic uptake of KPn infected neutrophils in-vitro. Importantly, treatment of KPn infected mice with necroptosis inhibitor reduced the bacterial burden and neutrophilia in-vivo in preclinical mouse model of KPn pneumonia. To our knowledge, this is the first report of inhibition of efferocytosis by KPn via modulation of cell death pathway, which may provide novel therapeutic targets.

O36 Gregory L. Kabachinski (AbbVie)
NETosis signaling is not conserved across species

Neutrophil extracellular traps (NETS) are expelled by neutrophils in a specialized form of cell death known as NETosis. Aside from efficiently killing microbes, NETs have been reported to be potent inducers of both the innate and the adaptive immune responses and observed in patients with auto-immune diseases, including lupus, vasculitis, and rheumatoid arthritis. Developing drugs to inhibit NETosis could be beneficial to a wide variety of patient populations. To date, the best characterized drug target for NETosis is peptidylarginine deiminase 4 (PAD4). We found that despite inhibitors blocking NETosis in human neutrophils, we show that neutrophils from PAD4-/- and PAD2-/- mice undergo NETosis to the same extent as wild type animals. Surprisingly, we found another essential singling pathways is required for NETosis in human neutrophils but not in mouse neutrophils. We conclude that the process of NETosis is not well conserved between species and that mouse disease models should be used with caution in understanding a links between NETosis and human disease.

O37 Oliver Söhnlein
Klinikum der Universität München, Munich, Germany
Kiss of death: neutrophils kill smooth muscle cells to induce atherosclerotic plaque destabilization

Because of the scarce detection of neutrophils in atherosclerotic plaques compared to other immune cells, their contribution to the pathogenesis of atherosclerosis has largely been neglected. However, in the last years studies have accumulated pointing towards the contribution of neutrophils to various stages of the disease. During this talk, I will address the stage-dependent importance of neutrophils in atherosclerosis and describe therapeutic implications derived from this knowledge.

O38 Mariana J. Kaplan
National Institutes of Health, Bethesda, USA
Neutrophils and their death in the pathogenesis of systemic autoimmune diseases

Autoimmune diseases are characterized by a breakdown of immune tolerance to self-driven by genetic/environmental interactions. Through immunomodulatory functions and dysregulated formation of neutrophil extracellular trap (NET), neutrophils from individuals with systemic autoimmunity may play a crucial role as source of externalized modified autoantigens. Oxidation of nucleic acids and posttranslational modifications of proteins distinctly occur during NET formation and may promote enhanced immunogenicity. Various autoantibodies, immune complexes, and other inflammatory stimuli promote NET formation in individuals with autoimmune diseases. Associations between dysregulated NET formation and NET clearance and adverse outcomes in systemic autoimmune diseases, including thrombosis, pregnancy complications, cardiovascular disease and renal damage have been reported. Various therapies that may modulate dysregulated neutrophil subsets and aberrant NET formation are being tested and may lead to novel characterization of targets critical for the induction and maintenance of autoimmune responses

O39 Peter A. Nigrovic
Harvard Medical School, Boston, USA
Modulation of neutrophil and megakaryocyte function by emperipolesis

Neutrophils are routinely observed within bone marrow megakaryocytes, an interaction termed emperipolesis. The nature and significance of emperipolesis is unknown. Using 3-D marrow imaging, *in vitro* modeling, and complementary *in vivo* validation, we find that emperipolesis is common and rapid, affecting multiple neutrophils that enter megakaryocytes and then egress intact. During emperipolesis, neutrophils may penetrate into the cytoplasm of host megakaryocyte to donate membrane that then emerges on megakaryocytes and their daughter platelets. Preliminary studies suggest further that neutrophils emerge altered, with enhanced migratory capacity both *in vitro* and *in vivo*. Emperipolesis is thus an intriguing cell-in-cell interface between immune and hematologic systems whereby passage of neutrophils through megakaryocytes functionally modulates the behavior of both lineages.

O40 Zvi Fridlender
Hadassah Medical Center, Jerusalem, Israel
Different sub-populations of cancer-related neutrophils, their activation and recruitment into tumors

In recent years, the role of neutrophils in cancer biology has been a matter of increasing interest. Tumor-associated neutrophils (TANs) account for a significant portion of the inflammatory infiltrate in many tumors. Over the past several years, we and others have demonstrated that TANs can have a dual role in tumor biology, and acquire either anti-tumorigenic or pro-tumorigenic functions. We have recently identified a heterogeneous subset of circulating low-density neutrophils (LDN) that accumulate continuously with cancer progression, and display immunosuppressive properties, in contrast to the mature, high-density neutrophils (HDN). We now aim to characterize the phenotype of the different neutrophil subsets in cancer patients, and understand the clinical implications of these neutrophil subpopulations in cancer. We find that LDN are significantly increased in advanced lung cancer patients, but not in early lung cancer or in chronic inflammation (COPD). Preliminary results suggest that high levels of LDN (10% or more) in the LD fraction, negatively correlate with survival in late stage patients. Whereas HDN display a mature morphology, LDN are composed of subsets of cells at different stages of maturation. HDN and LDN also differ in their ability to phagocyte and the cytokines they secrete. In addition, we have aimed to assess in animal models of lung cancer, the ability of circulating HDN and LDN to infiltrate the primary tumor and evaluate the mechanisms by which intra-tumoral neutrophils coordinate the migration of various immune cell types to lung primary tumors and metastatic lesions.
Proper understanding of the ways neutrophils support or fight cancer and affect tumor immune microenvironment will help us develop strategies to direct the immune system against the tumor.

O41 Stephen Renshaw
University of Sheffield, Sheffield, UK
Reverse migration as a therapeutic target in inflammation resolution

Neutrophils are the first immune cells recruited to a site of injury or infection, where they perform many functions. Having completed their role, neutrophils must be removed from the inflammatory site for restoration of normal tissue homeostasis. This can occur either by apoptosis and efferocytosis or by reverse migration away from the wound . Disruption of these tightly controlled physiological processes of neutrophil removal can lead to a range of inflammatory diseases. We used an in vivo zebrafish model to understand the role of lipid mediator production in neutrophil removal. Following tailfin amputation in the absence of macrophages, neutrophillic inflammation does not resolve. This is due to loss of macrophage-dependent handling of eicosanoid prostaglandin E2, which drives neutrophil removal via promotion of reverse migration. Knockdown of endogenous prostaglandin E synthase gene reveals PGE2 as essential for neutrophil inflammation resolution. Furthermore, PGE2 is able to signal through EP4 receptors during injury, causing an increase in Alox15 production, and switching towards anti-inflammatory eicosanoid signaling. Our data confirm regulation of neutrophil migration by PGE2 and LXA4 in an in vivo model of inflammation resolution. This pathway may contain therapeutic targets for driving inflammation resolution in chronic inflammatory diseases.

O42 Sven Brandau

Universitätsklinikum Essen, Essen, Germany

Identity of low-density neutrophils in cancer and their relation to MDSC

In the peripheral blood of patients with solid cancers a fraction of so-called low density neutrophils (LD-PMN), virtually absent in healthy blood donors, is significantly expanded. These LD-PMN have been demonstrated to inhibit the proliferation of T cells and are considered the equivalent of murine PMN-MDSC in the human peripheral blood.

We analyzed the frequency and suppressive capacity of several subsets of human circulating PMN-MDSC in patients with head and neck cancer (HNC) as well as urological cancers and compared their function with M-MDSC and e-MDSC, two other human MDSC subsets.

A high frequency of PMN-MDSC in the blood was strongly correlated with poor overall survival in HNC. T cell suppressive activity was higher in PMN-MDSC compared with M-MDSC and e-MDSC. A subset of CD66b+/CD11b+/CD16+ mature PMN-MDSC displayed high expression and activity of arginase I, and was superior to the other PMN-MDSC subsets in suppressing proliferation and cytokine production of T cells in both cancer types. High levels of this CD11b+/CD16+ PMN-MDSC, but not other PMN-MDSC subsets, strongly correlated with adverse outcome in HNC.

In this study we identified mature Arginase+/CD11b+/CD16+ PMN-MDSC as the human MDSC subset with the strongest immunosuppressive activity and the highest clinical relevance in two types of solid cancers. In contrast to common believe we also identified mature, and not immature cells, as the strongest MDSC component in human circulating LD-PMN. Our results will guide future immunomonitoring and functional analysis of circulating myeloid cells in patients with cancer.

O43 Karin Christenson

Göteborgs Universitet, Gothenburg, Sweden

Neutrophils in inflamed joints retain phenotypic characteristics of naïve blood cells

Objective: In inflammatory arthritis, leukocytes accumulate in the normally acellular synovial fluid of inflamed joints. In early disease and during flares, neutrophils dominate the inflamed fluid and transmigrate from circulation through the synovium before ending up in the inflamed joint. The transmigration process per se is believed to change the neutrophil phenotype, resulting in cellular priming and surface receptor rearrangements. We have investigated the phenotypic alterations of neutrophils migrating from blood to inflamed synovial fluid in patients with inflammatory arthritis.

Methods: Transmigrated neutrophils from synovial fluid of patients with inflammatory arthritis were collected and compared to neutrophils from peripheral blood. For comparison, transmigrated neutrophils from healthy donors were collected by controlled skin methods. Cell surface structures were evaluated by flow cytometry and cytokine content in cell free fluids measured by multiplex assay/ELISA.

Results: Synovial fluid neutrophils from patients with inflammatory arthritis displayed very limited signs of priming and were often comparable to those of corresponding blood neutrophils. Moreover, synovial fluid neutrophils could be further primed in vitro, in contrast to skin chamber/blister neutrophils that already displayed an activated phenotype. The synovial fluid mediated transmigration of unrelated peripheral neutrophils in vitro without receptor rearrangement.

Conclusion: Our findings indicate that transmigration can occur without priming and receptor rearrangements and suggest that chemoattractants (and chemotactic receptors) capable of biased signaling, i.e., that drives migration without inducing phenotypic changes, are involved in neutrophil transmigration to synovial fluid.

List of Abstracts (posters)

P1 CD62L AND CD36 DEFINE NEW SUBSETS OF LOW-DENSITY NEUTROPHILS IN CANCER PATIENT BLOOD

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Subsets of circulating neutrophils with T-cell inhibitory functions have been reported to increase with disease progression, in systemic inflammation defined as activated neutrophils CD62Ldim, or as low-density neutrophils (LDN) in cancer settings. LDN are neutrophils found within PBMCs after density gradient centrifugation instead of sedimenting in the granulocyte pellet as conventional normal-density neutrophils (NDN) do. LDN is a heterogeneous population of neutrophils, and the search for novel biomarkers is needed to identify subset(s) of LDN that have T-cell suppressive function and understand their origin and pathological role in cancer. The main goal of this study was to confront newly-identified density marker with activation status of neutrophils. We first decided to identify a marker specific to LDN in contrast to NDN. We used publicly available transcriptomic data of LDN and NDN isolated from peripheral blood of cancer patients. We identify CD36 as one of the most upregulated genes on LDN as compared to NDN. Using fresh blood from cancer patients, we found through FACS analysis, that CD36 was exclusively expressed by a subset of LDN, and not expressed at all on NDN. Similarly, we showed that neutrophil activation associated with CD62L downregulation (CD62Ldim) was specific to a subset of LDN. Interestingly, we found that CD62L and CD36 were not co-regulated and therefore both define new subsets of LDN. We are currently developing functional assays on the different subsets of LDN that we previously identified, to test their T-cell suppressive properties and other tumor-promoting functions.

P2 RESHAPING NEUTROPHIL FUNCTION IN CANCER

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Neutrophils are the most common immune subset and infiltrate most solid tumours. In addition to their tumour promoting functions such as tissue remodelling and angiogenesis, emerging evidence suggests that neutrophils can also have anti-tumour functions. Exploiting the potential anti-tumour function of neutrophils is an avenue for new cancer therapeutics. Existing cancer immunotherapies such as, checkpoint inhibitors, activate cytotoxic T cells but are only effective in a minority of patients and cancers. Clearly there is a clinical need to improve immunotherapeutic approaches to cancers. We hypothesize that harnessing anti-tumour function of neutrophils can reshape the tumour microenvironment leading to decreased tumour growth and providing an additional immune-based approach to treat cancers. Consistent with this hypothesis, administration of microbial bioparticles in Lewis Lung Carcinoma led to a substantial influx of neutrophils into tumours. Using mice which express the photoconvertible protein Kaede to distinguish neutrophils recruited into tumours following microbial stimulation. We demonstrated that recruited and tumour neutrophils had an activated phenotype characterised by upregulation of CD11b expression and downregulation of CD62L and CXCR2. This influx of activated neutrophils into tumours was paralleled by recruitment of activated T cells. Remarkably, tumour growth was significantly decreased as early as two days following treatment suggesting that neutrophils can play a significant role in controlling tumour growth. Taken together our results point to a substantial potential of modulating neutrophil function to reshape the tumour microenvironment and achieve tumour control.

P3 SPATIO-TEMPORAL DYNAMICS OF TUMOR-ASSOCIATED NEUTROPHILS IN SMALL TUMOR LESIONS

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Tumor-associated neutrophils (TAN) can influence tumor progression. This process is highly context-dependent and both, pro-tumor and anti-tumor effects of TAN have been described. We have used a novel genetic mouse model, termed CatchUp, for intravital tracking of migratory patterns of TAN in small tumor lesions. Using this model, we established methods to separately track migration of intratumoral versus peritumoral TAN in the same animal over time. Intratumoral TAN showed longer persistence and reduced motility compared with their peritumoral counterparts. Applying AZD5069, a pharmacologic blocker of CXCR2, inhibited early TAN entry into the tumor lesion. However, at later time points CXCR2 blockade had profound inhibitory effects on the peritumoral compartment. Our experimental model offers new insight into the spatial dynamics of TAN, enabling in vivo monitoring of neutrophil trafficking following pharmacologic intervention, and reveals distinct functionalities of intralesional versus peritumoral TAN that are partially and differentially regulated via the CXCR2 signaling pathway.

P4 PHENOTYPING OF CIRCULATING NEUTROPHILS IN HEPATOCELLULAR CARCINOMA

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Abundant evidence now clarifies a role for neutrophils as key immune mediators involved in promoting cancer progression/metastases. Hepatocellular carcinoma (HCC) is associated with a high blood neutrophil-to-lymphocyte ratio that predicts poorer survival. Our group has previously shown that neutrophil depletion in a murine HCC model has profound anti-tumour effects (Wilson CL et al. 2015). As neutrophils are also vital for host defence, pan neutrophil depletion is not a viable option for cancer patients already susceptible to infection. However, distinctive cancer-specific markers and functions are still poorly defined.
Neutrophil heterogeneity was assessed between healthy controls, liver cirrhosis and HCC patients by flow cytometry using FSC/SSC and the cell surface markers CD66b, CD11b and CD62L. Neutrophil function was assessed by measuring levels of reactive oxygen species (ROS) basally and in response to N-formyl-Met-Leu-Phe (fMLP) and platelet-activating factor (PAF). Neutrophil morphology/nuclear segmentation were assessed by cytospin and Giemsa staining. We observed functional differences in levels of activation markers CD11b (decreased) and CD62L (increased). Higher CD62L levels in HCC correlated with poorer performance status (p<0.001) and poorer survival. Moreover, we observed significantly lower levels of ROS in HCC both basally and in response to stimuli, which was associated with increased tumour size (p=0.039). This data suggests that there are phenotypic and biologically relevant changes in peripheral blood neutrophils from HCC patients. Further characterisation of these cells may enable us to better define the “pro-tumour neutrophil” associated with HCC and develop potential tailored therapeutics to target these cells selectively.

P5 POLARIZATION OF INNATE NEUTROPHILS DUE TO TOLLIP DEFICIENCY MODULATES COLONIC TUMORIGENESIS

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Cancer cells are known to modulate immune cells to cater their uncontrolled growth and advancement. However, the mechanism behind the cancer cell and immune cell communication/interaction is not well understood. In this study, we aim to identify the potential interaction of neutrophils with tumor cells and its underlying mechanisms. We have previously identified an inhibitory molecule, Toll-interacting protein (Tollip), in NFkB pathway which may be involved in the innate immune cell polarization. We employed the AOM and DSS model to mimic human colorectal cancer in mice, and studied the effect of neutrophils on murine colon cancer development as well as the role of Tollip in neutrophil polarization. We found that mice with Tollip knockout have consistently better clinical outcomes and lower polyp formation in the distal colonic region. Histology stainings and flow cytometry were performed to examine tissue markers for colon tumorigenesis and characterize neutrophil phenotypes in vivo. In vitro studies were done to investigate the cellular and molecular mechanisms behind the communication between cancer cell line, MCF10a, and primary neutrophils isolated from WT and Tollip deficient mice. We observed that Tollip deficient neutrophils exhibit a more potent ability to directly suppress tumor cell growth as compared to WT neutrophils. Elevated tumor cell apoptosis due to contact with Tollip deficient neutrophils may account for the tumor cell suppression. Mechanistically, selective miRNAs expressed by Tollip deficient neutrophils may be responsible for enhanced tumor cell apoptosis. Further research into characterizing the cellular and molecular pathways involved in communications between the cancer cell and neutrophils involving Tollip is needed and may hold promise for innate immune cell-based cancer therapies.

P6 IgA VS IgG: THE POTENTIAL AND DANGER OF IgA AS POTENT IMMUNE ACTIVATOR.

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Antibody-opsonized pathogens can activate immune cells via Fc receptors. Both IgA Fc receptor (FcαRI) and IgG Fc receptor IIA (FcγRIIA) are thought to initiate similar signaling pathways and responses, but we previously showed that only IgA triggering of neutrophils led to leukotriene B4 release with concomitant neutrophil migration. In this study we investigated cellular activation through IgA or IgG in more detail using different methods, including live cell imaging, (phospho)proteomics and metabolomics. No differences were observed in uptake of IgG- or IgA-coated beads and subsequent release of reactive oxygen species and neutrophil extracellular traps. However, crosslinking of FcαRI led to a slower but stronger and more sustained signaling profile, exemplified by increased intracellular calcium and phosphotyrosine levels. Only IgA stimulation induced downstream events, like release of cytokines, chemokines and pro-inflammatory lipids. Importantly, enhanced activation through FcαRI is not neutrophil specific, as stimulation of monocytes with IgA also led to increased activation. These results support 1) that signaling routes of FcγRIIA differ from those that are initiated by FcαRI, resulting in distinct functional profiles, and 2) IgA is a more potent activator of immune cells than previously anticipated. This may have significant implications in autoimmunity, as IgA auto-antibodies are found in a multitude of autoimmune diseases. Moreover, IgA may represent a potent novel immune activator during cancer immunotherapy.

P7 NOVEL CANCER IMMUNOTHERAPY, INTRATUMOURAL COMPLETE FREUNDS ADJUVANT (CFA) – DEPENDENCY ON NEUTROPHIL INFILTRATES.

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Our novel cancer immunotherapy involves an intratumoural injection of Complete Freund's Adjuvant (CFA), effectively turning the tumour into it's own vaccine. Since beginning a Phase I Clinical Trial at The Canberra Hospital assessing our treatment in multiple solid tumours, the current focus of our research is improving the potency of our novel anticancer immunotherapy and elucidating its precise mechanism of action using murine cancer models. We have studied CFA immunotherapy in 4 mouse models of cancer, with improved survival in mammary adenocarcinoma (4T1) and mastocytoma (P815). Complete regressions were seen in the P815 model after a single injection of 0.05mL of CFA. To elucidate the mechanism of action in this model we used our novel technique of 'fine-needle aspiration' (Carroll, et al. J Immunol Methods, 2015), to allow for sequential tumour analyses of infiltrating cells and retrospective correlation with survival. Our findings showed that mice with the longest survival had the highest leukocyte infiltrates. In particular, high neutrophil infiltrates at days 1-3 post treatment, and sustained neutrophil infiltrates were predictive of increased survival. We have investigated whether the efficacy of our cancer treatment can potentially be improved through manipulation of neutrophil infiltration and maturation. We have also endeavoured to identify the mechanism by which these neutrophils are eliciting their anti-tumour effects. From these findings, we can better understand how to improve our cancer treatments in patients and identify the cancer types that will derive the most benefit from our treatment.

P8 A PRO-TUMORAL ROLE OF TUMOR-ASSOCIATED NEUTROPHILS DURING THE COURSE OF CUTANEOUS CARCINOGENESIS

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A prominent role of neutrophils in cancer has been described. To which extend tumor-associated neutrophils (TAN) promote or inhibit cancer progression remains to be defined. We studied the role of TAN during cutaneous squamous cell carcinoma development. We performed comparative transcriptomic analyses of highly purified neutrophils infiltrating precancerous lesions, established tumors and skin surrounding these lesions, in a chemically-induced mouse skin carcinogenesis model (DMBA/PMA-treatment). We also set up an in vivo model consisting of the orthotopic grafting of a cutaneous squamous cell carcinoma cell line derived from DMBA/PMA-treated mice. Our work revealed a significant infiltration of neutrophils within lesions during the course of the chemically-induced skin carcinogenesis. We identified a unique gene expression profile of TAN within lesions compared to neutrophils isolated from their respective skin controls. The differentially expressed genes highlighted a pro-tumoral role of TAN during skin carcinogenesis progression. Consistent with this, specific depletion of neutrophils delayed tumor growth in the tumor grafting model. Further characterization of the contribution of these pro-tumoral tumor-associated neutrophils should provide valuable information for the treatment of cancer.

P9 MODULATION OF THE TUMOR AND METASTATIC IMMUNE MICROENVIRONMENT BY NEUTROPHILS.

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Tumor-associated neutrophils (TANs) make up a significant portion of the immune cell infiltrate in many cancers, but the mechanisms by which these cells affect tumor progression are only recently being investigated. TANs actively secrete cytokines and chemokines, modifying the recruitment and polarization of various immune cells in the tumor microenvironment. We aimed to evaluate the mechanisms by which neutrophils coordinate the migration of various immune cell types to lung primary tumors and metastatic lesions. TANs isolated from AB12 (mesothelioma) and LLC (adenocarcinoma) primary tumors were co-cultured with splenocytes using a Transwell assay, and recruitment of immune cells was assessed by Flow Cytometry. Recruitment was also assessed in vivo, using an ""air-pouch"" approach. Lung metastases were assessed using the metastatic LKR-M lung cancer model. The contribution of specific cytokines was demonstrated using neutralizing antibodies. We find that TANs isolated from primary tumors attract high amounts of monocytes and B cells. Preliminary data suggest that TNFalpha, but not CXCL12 and CXCL13, play a major role in B cell recruitment. In contrast, metastases' neutrophils display a different chemotactic profile, recruiting monocytes and CD4 T cells, but not B cells. Interestingly, it appears that the specific mechanism of recruitment of monocytes differ between the primary tumor and metastases.

Elucidating the chemotactic forces played by neutrophils during tumor and metastatic development will provide us with a deeper understanding of the ways these cells support or fight cancer, and ultimately help develop new strategies to direct the immune system against the tumor.

P10 HYPERGLYCEMIA IMPAIRS NEUTROPHIL MOBILIZATION LEADING TO ENHANCED METASTATIC SEEDING

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Diabetes patients are at an increased risk of being diagnosed with cancer and face poorer prognosis mainly due to increased metastatic spread. In previous studies, we have shown that neutrophils have the capacity to limit metastatic seeding. This knowledge, together with reports suggesting that neutrophil function is impaired in hyperglycemia, prompted us to test whether increased metastatic spread in diabetes is a result of hyperglycemia-induced neutrophil dysfunction. To this end we evaluated tumor growth and metastatic progression of 4T1 mammary tumor cells in Streptozotocin-induced hyperglycemic mice.

Our data show that while primary tumor growth is attenuated in hyperglycemia, metastatic progression is enhanced. Surprisingly, although neutrophil mobilization is impaired in hyperglycemic tumor bearing mice, neutrophil function remains intact. We find that reduced neutrophil mobilization in hyperglycemic mice during the premetastatic stage results in an increase in metastatic seeding. Finally, our data show that while normalizing glucose levels rescues primary tumor growth, it concomitantly restores neutrophil mobilization and reduces metastatic seeding in the lungs. Taken together, our results show that impaired neutrophil mobilization in hyperglycemic tumor bearing mice leads to an increase in metastatic seeding and therefore to a worse outcome.

Our work presents novel insights into the deleterious effect of hyperglycemia on immune function and how this affects tumor growth and metastatic progression. Our work further demonstrates the critical importance of managing blood glucose levels and has significant implications for cancer patients with underlying diabetes.

P11 TRPM2 MEDIATES NEUTROPHIL KILLING OF DISSEMINATED TUMOR CELLS

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Metastatic spread to distant organs is the final and lethal stage in cancer progression and is the primary cause of cancer-related mortality. Focusing on the events that precede the arrival of disseminated tumor cells, we found that neutrophils accumulate in large numbers in the pre-metastatic lungs. Stimulated by tumor-secreted factors, these neutrophils provide anti-metastatic protection by eliminating incoming tumor cells. Neutrophils form physical contact with tumor cells and induce tumor cell apoptosis. The main objective of this study was to examine what is the mechanism of the killing process. Our preliminary data show that in the presence of Catalase, neutrophil cytotoxicity is inhibited, suggesting the involvement of H2O2 in the killing process. To gain further insight into the mechanism of neutrophil cytotoxicity we explored the events downstream from H2O2 secretion. We now show that neutrophil-secreted H2O2 induces a lethal Ca2+ influx in tumor cells. These observations indicate the possible involvement of a tumor cell expressed Ca2+ permeable channel which is utilized by neutrophils to induce tumor cell death. We found that neutrophil cytotoxicity is mediated by TRPM2, a ubiquitously expressed H2O2-dependent Ca2+ channel. Indeed, TRPM2 knockdown or CRISPR mediated ablation of TRPM2 rendered tumor cells resistant to neutrophil cytotoxicity. Importantly, TRPM2 perturbation circulation tumor cells seeded more efficiently in the premetastatic lung. In summary, our study provides insight into the mechanism determining tumor cell susceptibility to neutrophil cytotoxicity, a mechanism which depends on the expression of the H2O2 responsive Ca2+ channel - TRPM2

P12 PROTEOMIC ANALYSIS OF NEUTROPHILS EXPOSED TO NANOGEL

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Objective: Nanogels are nanoparticles having many biomedical applications and can be chemically modified to transport drugs to specific tissues or cells, being a good candidate to carry anticancer drugs. PVP nanogels are good candidates for drug delivery systems since they are biocompatible and promote discrete neutrophil activation, so the aim of this study was to compare the neutrophil proteomic pattern after exposure to nanogels to quiescent controls. Methods: Neutrophils were exposed to nanogels particles, submitted to functional tests for neutrophil activation, morphology and viability, analyzed by light microscopy and NBT test. Aliquots of the cells exposed to nanogels and to HBSS buffer (control) were submitted to label free proteomic analysis using a Thermo Dionex UPLC and an Orbitrap Elite mass spectrometer. Regulated proteins were defined based on univariate and multivariate statistical analysis. Proteins were mapped to pathways and an in silico interactome was analyzed. Results: Proteomic analysis resulted in a total of 238 regulated proteins, being Leukocyte transendothelial migration, Chemokine signaling pathway and Regulation of actin cytoskeleton among the most represented pathways, as well as adhesion, motility and transmigration process were among the most represented groups.

Conclusion: Taken together, our findings show that neutrophils are modulated by PVP nanogels, showing a mild activation profile and interference in migration and endocytosis, that might improve the usage to deliver antitumoral agents via TINs.

P13 REGULATION OF ICAM-1 IN HUMAN NEUTROPHILS

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ICAM-1 is a member of the immunoglobulin superfamily known for its expression on the surface of endothelial cells, where it is required for leucocyte transmigration. In several inflammatory conditions ICAM-1 has also been found on the surface of neutrophils, and yet little is known about the function of neutrophil ICAM-1. This study seeks to investigate the role of ICAM-1 in essential neutrophil functions, such as phagocytosis, and further explore how ICAM-1 is expressed in inflammatory conditions. Peripheral blood-derived neutrophils were isolated from healthy donors by plasma-percoll gradients and ICAM-1 expression determined by flow cytometry. To measure neutrophil phagocytosis, uptake of heat-killed S. aureus was monitored in a whole blood assay. ICAM-1 expression was also measured in neutrophils obtained from the pleural fluid compartment of parapneumonic effusion and empyema patients. We show that TNF-alpha (n=3), LTA (n=1), and LPS (n=10) induce neutrophil ICAM-1 expression after 6-hour incubation. Furthermore, LPS induced ICAM-1 expression is also ablated after treatment with the translational inhibitor cycloheximide (n=3). Using the whole blood assay, we show that neutrophils expressing high levels of ICAM-1 phagocytose more S. aureus than ICAM-1 low populations (n=4). Neutrophils obtained from pleural fluid samples show dramatically increased levels of ICAM-1 expression compared to healthy donor blood neutrophils (n=5). In conclusion, these studies provide a unique insight into the role of neutrophil ICAM-1 and may allow for an improved understanding of how basic neutrophil functions are regulated and modified in inflammatory conditions.

P14 ANALYSIS OF CHEMOTAXIS IN HUMAN POLYMORPHONUCLEAR NEUTROPHILS (PMN) FROM CONTROLS AND PATIENTS WITH ACTIN DYSREGULATION

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PMN migrate along chemoattractant (CHX) gradients until cells reach a nidus of inflection/inflammation. Assays have been developed to assess chemotactic responses of PMN in vitro. While most are endpoint assays based on migration through a thin, porous membrane, EZ TAXIScan instrumentation temporally monitors the migration of PMN over longer distances. Two wells are separated by a slightly raised platform 260μm in length and covered by a coverslip (5μm depth). Cells are loaded into one well and then oriented along the edge of the platform. CHX is added to the other well to initiate migration, and images are acquired every 30 sec for an hour as PMN migrate across the platform. Using cell tracking software to analyze images, sequential positional coordinates of individual migrating cells can be determined as a function of time. Using this temporal data, movement of individual cells can be reconstructed. Since data are collected with time and position, multiple parameters can be derived – overall distance, directed distance (parallel to the CHX), random distance (orthogonal to the CHX), overall velocity, directed and random velocity vectors, and time-to-event analysis (number of cells completing migration and elapsed time). With buffer alone as CHX, directed and random velocity vectors are equivalent; with fMLF, there is a 4-fold increase in directed velocity vector without concurrent increase in random vector. PMN from patients with actin dysregulation (WDR1, RAC2) exhibit reduced overall velocity with fMLF. Videos reveal distinct morphological differences in the abnormal cells. Funded by NCI Contract No HHSN261200800001E.

P15 MULTI-ORGAN FAILURE OCCURS DURING CRITICAL ILLNESS AND IS MEDIATED IN PART BY DESTRUCTIVE NEUTROPHIL-TO-ENDOTHELIAL INTERACTIONS.

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The beta2 integrin receptor, CR3 (Complement Receptor 3; CD11b/CD18) binds endothelial Intercellular Adhesion Molecule-1 (ICAM-1) and plays a role in promoting the adhesion of activated neutrophils to inflamed endothelia which can cause vascular damage. Leukadherin-1 (LA-1) is a small molecule anti-inflammatory which acts as an allosteric activator of CR3. LA-1 has been shown to promote adhesion of blood neutrophils to inflamed endothelium and restrict tissue infiltration to sites of infection or injury. Therefore LA-1 offers a novel mechanism of anti-inflammatory action by activation, rather than inhibition, of the neutrophil CR3 integrin. Whether promotion of neutrophil:endothelial interaction by this novel therapeutic is of benefit or detriment to endothelial barrier function is not known. Neutrophils were isolated from critically ill septic and trauma patients, as well as healthy donors, and adhered to TNF-activated human umbilical vein endothelial monolayers in the presence or absence of fMLP and/or LA-1. Electric Cell-substrate Impedance Sensing (ECIS) was used to quantify endothelial barrier function and permeability. Neutrophils from critically ill trauma and septic patients caused endothelial barrier disruption which exceeded that caused by cells obtained from healthy controls. LA-1 protected barrier function even in the absence and presence of fMLP which served as a secondary stimulant. LA-1 protection was also observed by quantifying collagen exposure underlying endothelial cells challenged with fMLP-stimulated neutrophils. LA-1 treatment resulted in decreased migration dynamics of neutrophils crawling on an endothelial monolayer with reduced speed, path length and displacement. The CR3 agonist LA-1 protects endothelial barrier function from damage caused by neutrophils obtained from critically ill patients.

P16 NEUTROPHIL FUNCTIONALITY DIFFERS BY SHEEP BREED IN RESPONSE TO HELMINTH PARASITE INFECTION.

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Neutrophils are versatile cells that play important roles in immunological responses to various pathogens. Early innate immune responses to the helminth parasite Haemonchus contortus (Hc) varies between breeds of sheep. Parasite-resistant St. Croix (STC) sheep exhibit abomasal neutrophilia within the first 3 days of infection. This occurrence is delayed in parasite-susceptible Suffolk (SUF) sheep, indicating a relationship between neutrophil recruitment and development of full host protective responses. This study aimed to determine differences in chemotaxis, response to antigen (Ag) and effect on parasitic larvae between neutrophils from STC and SUF sheep in vitro. Neutrophils from Hc-primed STC and SUF sheep were plated on Matrigel-coated cell migration plates and exposed to Hc larval Ag (HcLA), IL-8 or complete media (CM). Total migrated cells were counted hourly using an Incucyte S3 cell imaging system for 24 hours. At 12 hours, the percentage of STC-derived neutrophils migrating towards HcLA was greater than SUF neutrophils (25% and 10%, P < 0.001) while cells from both breeds showed similar ability to migrate towards IL-8 (60% and 68%). Interestingly, 25% of neutrophils displayed migration in the absence of stimulation compared to only 7% of STC neutrophils (P < 0.001). After HcLA exposure, neutrophils from STC and SUF formed extracellular traps at a 35-fold higher rate than CM controls determined by sytox assay (P < 0.001), however, larval ATP measured after culture with STC and SUF neutrophils indicated that STC cells induced increased larval morbidity compared to SUF neutrophils (0.05 μM vs 0.1 μM ATP, P < 0.001). Taken together these data indicate enhanced ability of STC-derived neutrophils to migrate in response to larval Ag and document impairment of SUF neutrophils response to helminth parasites.

P17 ACUTE PHASE SEROTONIN MEDIATED NEUTROPHIL TRAFFICKING IS INDEPENDENT OF ENDOTHELIAL ADHESION MOLECULES

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In a mouse strain deficient for tryptophan hydroxylase 1 (Tph1-/-), the rate limiting enzyme for serotonin synthesis, neutrophil-endothelial interactions are dampened during acute inflammation. It is not completely understood if this 5-HT triggered effect is directed towards circulating- and/or endothelial cells. Endotoxic shock was induced by i.p. administration of LPS. After 4 hours mice were sacrificed to obtain blood, abdominal aortas, and peritoneal fluids. Leukocyte adherence was also evaluated in a model of mesenteric ischemia/reperfusion injury. Following endotoxic shock blood neutrophils and monocyte subsets were similarly reduced in WT and Tph1-/- mice. We found 2.6±0.5 (x105) transmigrated Ly6Clow monocytes per mL peritoneal lavage in WT and 2.7±0.8 (x105 cells/mL) in Tph1-/- mice. In contrast, peritoneal neutrophil content was significantly reduced in KO mice (10±1 vs 19±3 x105 cells/mL in WT). Flow cytometric evaluation of endothelial ICAM, VCAM, P- and E-selectin expression revealed no relevant differences between the two groups. Circulating neutrophils however, showed a 40% reduced CD11b exporession in Tph1-/- mice, whereas PSGL-1, and LFA-1 were similar expressed. After 20 minutes of reperfusion following mechanically induced mesenteric ischemia neutrophil adhesion was dampened in Tph1-/- mice when compared to WT (19±1 vs 52±9 cells/ 0.04mm2) and circulating platelet neutrophil complexes (PNCs) were reduced by 40% in KO mice. Serotonin triggered neutrophil recrutiment and transmigration during acute inflammation as a consequence of bacterial infection or reperfusion following mesenteric ischemia. This effect seemed to be directed to blood neutrophils and was independent of endothelial adhesion molecules.

P18 AGE-RELATED DECLINE OF THE ACUTE LOCAL INFLAMMATION RESPONSE: A MITIGATING ROLE FOR THE ADENOSINE A2A RECEPTOR.

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Aging is accompanied by an increase in markers of innate immunity. How aging affects neutrophil functions remains of debate. The adenosine A2A receptor (A2AR), essential to the resolution of inflammation, modulates neutrophil functions. We sought to determine whether or not A2AR protects against the effects of aging. We monitored neutrophil influx, viability, and activation as well as cytokine accumulation in wild-type (WT) and A2AR-knockout mice (KO) at three different ages. Several readouts decreased with aging: neutrophil counts in dorsal air pouches (by up to 55%), neutrophil viability (by up to 56%), elastase and total protein in exudates (by up to 80%), and local levels of cytokines (by up to 90%). Each of these parameters was significantly more affected in A2AR-KO mice. CXCL1-3 levels were largely unaffected. The effects of aging were not observed systemically. Preventing neutrophil influx into the air pouch caused a comparable cytokine pattern in young WT mice. Gene expression (mRNA) in leukocytes was affected, with CXCL1 and CCL4 increasing and with TNF and IL-1α decreasing.
Conclusion. Aging has deleterious effects on the acute inflammatory response and neutrophil-related activities, and defective migration appears as an important factor. A functional A2AR signaling pathway delays some of these.

P19 ANALYSIS OF LEUKOCYTE TRANSEPITHELIAL MIGRATION USING A NEW IN VIVO MURINE COLONIC LOOP MODEL

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A characteristic feature of ulcerative colitis that is closely linked to clinical symptoms is a massive and dysregulated influx of neutrophils (PMN) across colonic epithelium. While molecular mechanisms controlling leukocyte migration across vascular endothelium have been extensively characterized in vivo, details of leukocyte transepithelial migration (TEpM) into the intestine remain poorly elucidated. Furthermore, current paradigm of PMN TEpM is based largely on in vitro studies due to the lack of suitable experimental animal models. Here we describe a new murine model of PMN intestinal trafficking that utilizes a vascularized proximal colonic segment (pcLoop). This model enables qualitative and quantitative determination of levels of PMN TEpM into the colonic lumen as well as assessment of PMN that remain associated with the epithelium or within the subepithelial space (lamina propria) in response to intraluminally administered chemoattractants. Consistent with previous in vitro studies, intraluminal injection of inhibitory antibodies against the leukocyte integrin CD11b/CD18 significantly increased subepithelial PMN accumulation and reduced trafficking into the colonic lumen. We extended these studies to determine contributions of epithelial tight junction-associated protein Junctional Adhesion Molecule-A (JAM-A) to PMN TEpM. We found that loss of JAM-A resulted in reduced PMN migration into the colonic lumen and subepithelial accumulation of PMN in intestinal epithelial-targeted JAM-A deficient mice. These findings highlight a novel role for epithelial JAM-A in regulating PMN TEpM in vivo and demonstrate the utility of this model for identifying receptors that may be targeted in vivo to reduce pathologic intestinal inflammation.

P20 CD47 REGULATES CD11b-DEPENDENT NEUTROPHIL TRANSEPITHELIAL MIGRATION DURING INTESTINAL INFLAMMATION.

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Excessive neutrophil (PMN) transepithelial migration (TEpM) to the intestine results in disease flares in individuals with ulcerative colitis. While it is known that PMN integrins such as CD11b/CD18, and the ubiquitously expressed glycoprotein CD47 play crucial roles in TEpM, mechanisms regulating this process remain uncharacterized. We therefore hypothesized that CD47 modulates PMN trafficking in the gut by regulating CD11b/CD18 function. Using in vivo ileal loop assays, we observed reduced LTB4 driven PMN TEpM in CD47-/- mice. Tissue specific deletion of CD47 in vivo and in vitro chemotaxis assays revealed that PMN-expressed but not epithelial expressed CD47 plays a key role in regulating TEpM. In addition, blocking CD11b/CD18 reduced in-vitro migration of CD47 expressing PMN to the levels observed with PMN from CD47-/- mice, suggesting CD47 regulates integrin-dependent chemotaxis. Co-immunoprecipitation and proximity ligation assays revealed close association of CD47 with CD11b in PMN. Consistent with defective migration, there was decreased CD11b upregulation upon chemoattractant stimulation in PMN from CD47-/- mice. Furthermore, we observed that integrin activation, as determined by antibodies that specifically bind to active conformations of CD11b/CD18, was reduced in CD47 deficient HL60 cells. Taken together, these findings provide strong evidence for CD47-mediated regulation of CD11b integrin function in PMN. Targeting CD47 may thus provide new therapeutic approaches to target dysregulated PMN infiltration in the intestine.

P21 NRF2 ACTIVATION DOWNREGULATES NEUTROPHIL OXIDATIVE BURST AND AFFECTS MIGRATION

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Polymorphonuclear neutrophils (PMN) are the first immune cells to be mobilized at the early phases of inflammation. Nrf2 (NF-E2-related factor-2) transcription factor regulates oxidative stress and also represses inflammation. In this study, we aimed to investigate the role of Nrf2 in the regulation of PMN various functions such as oxidative burst, NETosis, migration, cytokine release and phagocytosis. PMN were isolated from C57BL/6 wild type (WT) and Nrf2 knock out (KO) mice bone marrow using MACS beads. Each function was evaluated both at basal state and after ex vivo activation with appropriate stimuli. In some experiments PMN were pretreated with sulforaphane (Nrf2 inducer). We found that Nrf2 was able to decrease zymosan-induced PMN oxidative burst; sulforaphane-induced Nrf2 hyperexpression confirmed its implication. tnf-alpha gene transcription was decreased in zymosan-stimulated Nrf2 KO PMN, suggesting a role for Nrf2 in tnf gene regulation. Spontaneous migration of Nrf2 KO PMN was lower than that of WT PMN. Moreover, in response to low concentrations of CXCL2 or CXCL12, Nrf2 KO PMN migration was decreased despite similar CXCR2 and CXCR4 expression in PMN from both genotypes. Nrf2 thus seems to be required for an optimal migration. Finally, we revealed that Nrf2 does not seem to be involved in netosis nor phagocytosis. Altogether, these results suggest that Nrf2 could participate to oxidative burst downregulation and to an adequate migration. This study points out Nrf2 implication in PMN biology and may provide insights in the field of inflammation.

P22 ARAP3-MEDIATED INTEGRIN INACTIVATION PROMOTES NEUTROPHIL RECRUITMENT TO SITES OF INFLAMMATION

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Integrins are required for neutrophil recruitment. Contrasting with integrin activation, mechanisms regulating inactivation, and the function of integrin inactivation during neutrophil recruitment remain largely obscure. We tested here whether and how the dual Arf6 and RhoA GTPase activating protein (GAP) ARAP3 promotes integrin inactivation, and whether this regulates neutrophil recruitment. Neutrophil responses were analysed in response to integrin stimulation in vitro. Integrin distribution, ligand binding and activity status were analysed focussing on a5b1. Integrin activity and trafficking were also analysed in CHO reporter cells that express human aIIbb3. Neutrophil recruitment was addressed in response to peritonitis and acute lung inflammation (ALI). Endogenous integrins (and heterologous human aIIbb3) were activated in Arap3-/- neutrophils and ARAP3 kd CHO cells. ARAP3-mediated integrin inactivation affected a range of neutrophil functions. It enabled efficient neutrophil neutrophil chemotaxis and transendothelial migration in vitro and promoted efficient neutrophil recruitment to sites of inflammation, including in a model of acute lung inflammation in vivo. Our data identifies a function of the dual GAP ARAP3 in the regulation of integrin inactivation. Mechanistically, differential integrin activity is due at least in part to ARAP3-dependent regulation of integrin trafficking. Functionally, integrin inactivation is required for the efficient recruitment of neutrophils to sites of inflammation; in the inflamed lung this is due to a requirement for integrin inactivation in extravasation. More widely, our work is a rare demonstration of a protein to negatively regulate integrins and a first identification of any GAP to regulate integrin inactivation."

P23 A NOVEL CIS INTERACTION BETWEEN L-SELECTIN AND PECAM-1 DRIVES NEUTROPHIL TRANSENDOTHELIAL MIGRATION (TEM).

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Although the individual contribution of many cell adhesion molecules have been identified and characterised in regulating neutrophil TEM, very little is known about how their actions are collectively orchestrated in space and time. By subjecting primary human neutrophils and HL-60 cells to flow assays and state-of-the-art microscopic techniques (FRET/FLIM/iSIM/confocal/timelapse), we expose a newly discovered cis interaction between L-selectin and platelet-endothelial cell adhesion molecule (PECAM-1). Neutrophil L-selectin/PECAM-1 co-clustering is unilaterally driven by PECAM-1, and occurs exclusively during TEM. Interestingly, this co-clustering behaviour is specific to TEM across TNF-alpha- but not IL-1beta-stimulated endothelial monolayers. L-selectin/PECAM-1 co-clustering promotes ectodomain shedding of L-selectin during TEM, which in turn optimises TEM speed. Moreover, blocking p38 MAPK (downstream of PECAM-1 clustering), neutrophil-derived PECAM-1, or L-selectin shedding delayed the TEM time by 30%. The cytoplasmic tails of L-selectin (for ezrin-radixin-moesin binding) and PECAM-1 (Y663 and Y686 of the immune receptor tyrosine inhibitory motif) were both required for co-clustering, ectodomain shedding of L-selectin and optimal TEM speed. This is the first report showing a direct involvement of L-selectin in regulating neutrophil TEM. Co-clustering of L-selectin with PECAM-1 therefore triggers a unique intracellular signal, during TEM, that informs neutrophils of their exact location within the multi-step adhesion cascade. Small nucleotide polymorphisms within PECAM-1 that predispose cardiovascular risk will be discussed in light of co-clustering with L-selectin and TEM.

P24 TAMOXIFEN INHIBITS CHEMOKINESIS IN EQUINE NEUTROPHILS

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Neutrophils are terminally differentiated innate effector cells at the first line of host defense. Neutrophil migration within tissues is complex and involves several steps, during which these cells must be able to interpret a variety of chemical and physical signals. Exacerbated neutrophil activity can be harmful to surrounding tissues; this is important in a range of diseases, including equine asthma. Tamoxifen (TX) is a non-steroidal estrogen receptor modulator with effects on cell growth and survival. Previous studies showed that TX treatment in horses with induced acute pulmonary inflammation promoted early apoptosis of blood and BALF neutrophils, reduction of BALF neutrophil content, and improvement in animals' clinical status. Further, TX dampens chemotactic index and respiratory burst production in vitro. The aim of this study was to provide information on the effect of TX on chemokinesis in peripheral blood neutrophils from five healthy horses. Results showed that neutrophils increased migration and travelled distance in response to IL-8; but in the presence of TX, IL-8 did not produce neutrophil migration. This suggests that TX has an inhibitory effect on the kinesis of equine peripheral blood neutrophils stimulated with IL-8. However, further studies are required to fully understand the signaling pathways of TX on neutrophil chemokinesis."

P25 20-OH- AND 20-COOH-LEUKOTRIENE B4 INHIBIT LEUKOTRIENE B4-INDUCED NEUTROPHIL RESPONSES

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Leukotriene (LT) B4 is recognized as an excellent promoter of host defense. LTB4 induces the recruitment of neutrophils, the release of antimicrobial peptides and potentiates the ingestion and killing of pathogens. However, in humans, LTB4 has a short half-life and is metabolized into 12-oxo-LTB4 by monocytes/macrophages, or into 20-OH- and 20-COOH-LTB4 by neutrophils. Although LTB4 metabolites bind to the BLT1 receptor with high affinity, they poorly activate neutrophils, suggesting they might interfere with BLT1-mediated responses. The aim of our study was to determine the impact of LTB4 and its metabolites on human neutrophil functions. Neutrophils were isolated from the blood of healthy volunteers, then treated with LTB4 and/or LTB4 metabolites. 20-OH-LTB4 and 20-COOH-LTB4 were significantly less efficient than LTB4 to induce alpha-defensin-1 release by human neutrophils. These metabolites inhibited the LTB4-mediated migration and leukotriene biosynthesis and reduced BLT1 surface expression, suggesting that the mechanism by which LTB4 metabolites impair LTB4 is by desensitizing the BLT1. Using LTB4-alkyne, which cannot be metabolized into 20-OH- or 20-COOH-LTB4, we found that LTB4 is a chemotactic rather than a chemokinetic substance as initially described. Finally, inhibiting the degradation of LTB4 into 20-OH-LTB4 in neutrophils amplified LTB4-induced ERK phosphorylation and attenuated the chemokinetic effect of LTB4. Our data indicate that LTB4 metabolites act as natural inhibitors of LTB4-mediated responses and that preventing LTB4 metabolism might enhance some functions of neutrophils, supporting the idea that preventing LTB4 degradation might enhance host defense.

P26 SIGNAL ADAPTATION CONTROLS NEUTROPHIL SWARMING DYNAMICS AT LOCAL SITES OF INFLAMMATION AND INFECTION.

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Neutrophils are critical effector cells of the innate immune system that protect the host by migrating to inflammatory sites and tissue damages. Once outside the vessel, individual neutrophils show strikingly coordinated, extremely fast chemotaxis and swarm-like migration patterns, referred to as 'neutrophil swarming'. It is now clear that intercellular communication among neutrophils amplifies their recruitment in a feed-forward manner. However, it is completely unknown how the neutrophil swarming response is terminated. Mechanisms that stop neutrophil swarming might be either neutrophil-intrinsic or driven by external signals released by other innate immune cells. Using 2-photon intravital microscopy and elaborate conditional knockout strategies in mice, we identified G-protein coupled receptor (GPCR) desensitization by G-protein coupled receptor kinases (GRKs) as neutrophil self-regulating mechanism to stop neutrophil swarming. By generating primary neutrophils lacking all four expressed GRK family members, we could show that GRKs are essential for adapting neutrophil dynamics to high local concentrations of swarm-mediating chemoattractants. GRK-mediated signal adaptation was relevant for the formation of both persistent and transient neutrophil swarms. Our novel findings suggest a key role of GRK-mediated signal adaption for the termination of neutrophil swarming during inflammation and infection.

P27 NEUTROPHIL MIGRATORY CAPACITY IS ALTERED BY PASSAGE THROUGH THE CYTOPLASM OF MEGAKARYOCYTE IN EMPERIPOLESIS

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BACKGROUND: Emperipolesis (EP) is a common but poorly-understood phenomenon wherein bone marrow megakaryocytes (MKs) encompass intact neutrophils (PMNs). The physiological significance of EP is unknown.

METHODS: We assessed the frequency of EP in mice in experimental inflammation (peritonitis, arthritis). A model of EP was developed through incubation of cultured MKs together with PMNs, and characterized using electron microscopy and live-cell spinning-disk imaging. The impact of EP on PMN migration was interrogated in vitro using transwells, and in vivo using engraftment of labeled PMNs co-cultured with MK into inflamed mice.

RESULTS: EP is observed in 5% of MKs at baseline and increases 2-3-fold during inflammation. PMNs penetrate actively into MKs, entering within a vacuole (termed here the emperisome) followed by penetration into the MK cytoplasm. During this process, PMNs take up MK exosomes discharged into the emperisome. Following donation of membrane to platelets through fusion with the MK demarcation membrane system, PMNs exit alive and intact, demonstrating augmented migratory capacity to inflammatory sites compared with naïve PMNs.

CONCLUSIONS: We identify EP as a novel cell-in-cell interaction that mediates bidirectional biomass transfer between PMNs and MKs and that can enhance PMN migration to sites of peripheral inflammation.

P28 SEX DIFFERENTIALLY AFFECTS NOX-DEPENDENT AND -INDEPENDENT NETOSIS.

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As part of the immune response, neutrophils release extracellular traps (NETs) made of DNA complexed with antimicrobial proteins to trap invading microbes. However, adverse effects of NETs have been reported in multiple pathologies where sex differences have also been documented. Both NETs and the female sex have been associated with worse disease outcomes. As such, we aimed to investigate the existence of a sex-specific difference in NET formation (NETosis). We hypothesized that NETosis levels are elevated in females.

We isolated neutrophils from healthy men and women and compared NETosis levels between the two groups. NETosis was induced by the NADPH-oxidase (NOX)-dependent stimulus phorbol 12-myristate 13-acetate (PMA) and the NOX-independent stimulus calcium ionophore A23187. Fluorescently-tagged extracellular DNA was used as a proxy for NET release. We further detected for the expression of NET-associated proteins by immunofluorescence under a spinning disk confocal microscope.

Our data shows increased levels of NETosis in neutrophils isolated from females compares to males when stimulated with PMA (p<0.05), but lower NETosis in female-derived neutrophils stimulated with A23187 (p<0.05). In line with the lower DNA release observed in NOX-independent NETosis, immunofluorescence revealed lower levels of DNA decondensation and histone citrullination in neutrophils from females in the first hour post stimulation with A23187.

We conclude that there is a difference in the level of NETosis in neutrophils isolated from males and females, and that the type of stimulus is important to consider when studying these sex-specific differences.

P29 NEUTROPHIL EXTRACELLULAR TRAPS AND THEIR IMPLICATION IN RADIATION RESISTANCE

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PURPOSE: The protein High Mobility Group Box-1 (HMGB1), a key player in radioresistance is passively released from dying tumor cells and mediates responses to injury and inflammation. Recently, HMGB1 was found to be a component of neutrophil extracellular traps (NETs), which have been shown to facilitate tumor progression, promote metastasis and exhibit adverse effects in surgical stress. The objective of this study was to investigate the impact of HMGB1 on NETs formation and its relationship with radioresistance.

METHODS: In vitro: Human neutrophils were stimulated with recombinant HMGB1 (rHMGB1), 3%RPMI, PMA and NETs were quantified through Sytox Green fluorescence. In vivo: Murine bladder cancer cell line (MB49) were subcutaneously implanted into flanks of C57BL/6 and NETosis deficient PAD4-/- mice. Intraperitoneal injections of Glycyrrhizin (GLZ) was used to inhibit HMGB1 and intramuscular injections of DNAse was used to deplete NETs. Tumors were irradiated 2x5Gy and were followed till endpoint (1.5cm3).

RESULTS: In vitro: incubation of neutrophils with 50ng rHMGB1 significantly induced NETs formation compared to controls (p<0.0001) and this was reversed through addition of GLZ (p<0.0001). Our in vivo results demonstrate that PAD4-/- mice treated with GLZ showed delayed tumor growth kinetics (p=0.023) and increased overall survival post radiation (p=0.0231) compared to C57BL/6 + GLZ. Using a clinically relevant NETs deficient model, similar results were observed. C57BL/6 mice treated with DNAse and GLZ also showed a delay in tumor growth kinetics post radiation (p<0.0001).

CONCLUSION: Our results suggest that HMGB1 induces radioresistance via NETs formation. Clinically targeting the interaction between HMGB1 and NETs may improve overall response to radiation therapy.

P30 DIROFILARIA IMMITIS MICROFILARIAE AND THIRD STAGE LARVAE INDUCE CANINE NETOSIS RESULTING IN DIFFERENT TYPES OF NETS

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Dirofilariosis or canine heartworm disease is a zoonotic vector-borne disease caused by the nematode Dirofilaria immitis. In most cases, D. immitis infections cause chronic disease that may end-up fatally in a right heart failure and Cor pulmonale. The role of polymorphonuclear neutrophils (PMN) in early immune reactions against D. immitis stages has been scarcely investigated. NETosis has already been described to occur in response to other metazoan parasites highlighting the ability of NETs to entrap and sometimes kill motile and extreme large-sized pathogens The objective of the present studies was to analyse vital D. immitis microfilariae and third-stage larvae (L3) originating from infected mosquitoes for their capacity to induce NETs in canine PMN. For NET-related experiments canine PMN were exposed to D. immitis microfilariae or L3 in a time- and dose-dependent manner. NET formation was measured by quantification of extracellular DNA via Sytox orange®-mediated fluorescence intensities. Co-localization of extracellular DNA with NETs markers was visualized by immunofluorescence D. immitis microfilariae induced NET extrusion by canine PMN in a time but not dose-dependent manner. Microfilarial stages mainly induced spread and diffuse NETs while L3 additionally triggered aggregated NET formation. Neither NETosis nor entrapment was affected by DPI treatments suggesting that this event is NOX-independent. We here obtained new insights into the early host innate immune response against D. immitis demonstrating for the first time the capacity of microfilariae and L3 to induce NETs in the canine system. This effector mechanism of PMN might facilitate the firm entrapment and further killing of larvae by other leukocytes circulating in blood.

P31 NETs RELEASE IS ASSOCIATED WITH LUPUS OUTCOMES AND WEIGHT GAIN IN LUPUS-PRONE B6.MRL/lpr MICE

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Systemic lupus erythematosus (SLE) is an immune complex (IC)-mediated autoimmune disease where reactive oxygen species (ROS) produced by neutrophils cause oxidative damage and participate in the disease physiopathology. SLE also correlates with obesity. We have reported that weight gain accelerates the SLE onset in lupus-prone B6.MRL/lpr mice, which is preceded by an increase in ROS production by circulating neutrophils (FRBM 2015;86:362-73). Other neutrophil effector functions contribute to SLE outcomes, but it remains unclear which role neutrophil extracellular traps (NETs) play in SLE onset/outcomes. This study aims to examine whether NETs release is associated with SLE onset/outcomes and/or weight gain in female lupus-prone B6.MRL/lpr and control (C57BL/6) mice. Four-week-old mice were fed standard (SD) or cafeteria diet (CD), and the SLE onset and/or outcome was determined based on disease-specific clinical conditions and serum levels of antinuclear antibodies. At the end of 8, 12 and 16 weeks – when most animals had no clear signs of SLE, were in pre-lupus state, or had typical laboratory and clinical findings of SLE, respectively –, the percentage of weight gain was calculated and blood was collected. Serum levels of NETs were measured by ELISA. Comparison between 16-week-old B6.MRL/lpr mice fed CD and SD evidenced that NETs release: (i) increased after the SLE onset; (ii) was directly correlated with weight gain; (iii) was inversely correlated with ROS production by neutrophils stimulated with IgG-IC opsonized or not with complement. In conclusion, NETs release is directly associated with SLE outcomes and weight gain in B6.MRL/lpr mice, but it is inversely associated with ROS production mediated by IgG and complement receptors in neutrophils.

P32 NEUTROPHIL EXTRACELLULAR TRAPS INTYPE 1 DIABETES

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Neutrophil extracellular traps (NETs) were demonstrated to be an effective defence mechanism against infections, being able to entrap and eliminate various pathogens. These structures, composed of decondensed chromatin and antimicrobial proteins, also have the capacity to stimulate other cell subsets, for instance macrophages and dendritic cells (DC). Additionally, NETs are implicates in several autoimmune diseases, such as lupus erythematodes, vasculitides or type 1 diabetes (T1D) but their contribution to pathogenesis is elusive. In T1D, insulin-producing pancreatic beta cells are destroyed by autoreactive T lymphocytes. Even though innate immunity involvement is also clearly demonstrated in the development of T1D, its exact mechanism still remains unclear. To examine how neutrophils contribute to the genesis of T1D, we investigated the effect of NETs on DC function in T1D patients. We found that NETs influence mDC phenotype differently between patients and controls, but not pDC phenotype. They also triggered T1D DCs to release inflammatory cytokines – IFNα and IL-1β. On the contrary, T1D patient DCs primed with NETs (NET-DCs) produced lower level of IL-10. NET-DCs induced more IFNγ-producing CD4+ T lymphocytes and less T regulatory lymphocytes (Tregs) compared to controls. In addition, patients NET-DCs-induced Tregs expressed a low level of IL-2Rα, which implies their impaired function. Moreover, T1D-NETs contain more DNA and less antimicrobial peptides. So not only NETs differ between T1D and controls in effect on DCs, but also in their composition. Our findings shed light on the involvement of dendritic cells in pathogenesis of T1D, through their interaction with neutrophils.

P33 PATHOGENIC ENTAMOEBA HISTOLYTICA INDUCE NET FORMATION INDEPENDENTLY OF PKC AND ROS

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Entamoeba histolytica is a protozoan parasite and the causative agent of amoebiasis. Clinical manifestations of amebic infection range widely from asymptomatic to severe symptoms, including dysentery and extra-intestinal mainly liver abscesses. The neutrophil is the first cell of the host immune system to interact with the invading ameba, and it has been shown to play a protective role in the early host response to amebic infection of the liver. Pathogenic amebae seem to induce lysis of neutrophils releasing toxic substances that can kill amoebas as well as damage host tissue. Contrary to this early view, we have found that upon interaction with E. histolytica trophozoites, human neutrophils released NET that covered amoebas and reduced amoebic viability. We have now explored the molecular mechanism induced by amoeba to activate NET formation. Pathogenic E. histolytica activated in human neutrophils a signaling pathway involving the Raf/MEK/ERK pathway, but independently of PKC activation and ROS formation. Also amoeba-induced NET formation was much faster and stronger than PMA-induced NET formation. These data clearly show that NETosis induced by pathogenic amoebas uses a different mechanism from the one activated by PMA, and strongly suggest that NETs are an important defense mechanism to prevent the spreading of amoeba infection.

P34 TIMING OF REACTIVE OXYGEN SPECIES INVOLVEMENT IN FORMATION OF NEUTROPHIL EXTRACELLULAR TRAPS

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Neutrophils release extracellular traps (NETs), lattices of chromatin associated with antimicrobial proteins, in response to various stimuli. NETs are proposed to contribute to host defence by trapping and killing micro-organisms. However, they may also cause tissue damage or promote autoimmune reactions. It has been shown that activation of the NADPH oxidase is required for the formation of NETs in response to a large number of stimulants. The aim of this study was to examine the timing of oxidant production required for NET formation. Neutrophils were stimulated with phorbol myristate acetate or microbial stimuli and NET formation was assessed by Sytox green plate assay and microscopy. The NADPH oxidase inhibitor diphenyleneiodonium (DPI) was added either prior to stimulation or at specified times afterwards. Addition of DPI 60 min after stimulation was still able to inhibit NET formation, suggesting the initial oxidative burst was not sufficient and that a functional NADPH oxidase is required for an extended period. Addition of H2O2 immediately after the DPI was added at 60 min post stimulation partially enabled NET formation. In contrast, H2O2 added 60 min after stimulation was unable to promote NET formation when DPI had been added prior to neutrophil activation. These results indicate that an early oxidative burst combined with oxidant production after 60 min are required for optimal NET formation. The signal to form NETs may require an accumulation of oxidative changes over an extended period or an oxidant-dependent event may occur late in the activation process.

P35 MOLECULAR MECHANISMS OF BOVINE NETOSIS INDUCED BY APICOMPLEXAN PARASITES

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Toxoplasma gondii and Besnoitia besnoiti are obligate intracellular apicomplexan parasites which were demonstrated as potent inducers of neutrophil extracellular traps (NETs) formation. Via this innate effector mechanism, neutrophils are capable of entrapping tachyzoites, thereby immobilizing them and potentially preventing them from host cell invasion. Thus, NETosis may play an important role in outcome of toxoplasmosis/besnoitiosis in the in vivo situation. Current study analysed T. gondii- and B. besnoiti-tachyzoites-triggered formation of NETs and ROS production in the bovine system. The signalling pathways of store-operated calcium entry (SOCE), MAPK, and autophagy were also evaluated through pharmacological approaches. In addition, we evaluated if T. gondii- and B. besnoiti-infected endothelium induces NETosis. T. gondii-tachyzoites were co-cultured with freshly isolated bovine polymorphonuclear neutrophils (PMN) and NETs formation was evaluated by quantification of extracellular DNA and microscopy. ROS production was estimated using fluorescent probes. For infected endothelium experiments, primary BUVEC cells were infected with either T. gondii- or B. besnoiti-tachyzoites and 12 and 18 h p. i formation of NETs was evaluated. We here show that T. gondii and B. besnoiti are capable to trigger NETosis. Overall, tachyzoite-induced NETosis proved dependent on both, intracellular Ca++- concentration and MAPK-related pathways. Finally, infected endothelium also induces release of NETs giving new insights on how this process can occur in vivo as demonstrated for other pathogens.
We here confirmed apicomplexan tachyzoites as potent inducers of NETosis and added some details on molecular mechanisms of parasite-triggered NETosis.

P36 NEUTROPHIL EXTRACELLULAR TRAPS AND TYPE 1 IFN CONTRIBUTE TO AUTOIMMUNITY IN HIDRADENITIS SUPPURATIVA

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Hidradenitis suppurativa (HS) is a neutrophilic inflammatory skin disorder with an unknown etiology primarily affecting intertriginous areas. Considering the predominant cellular infiltrate, we sought to understand the role of neutrophil extracellular traps (NETs) in HS. In peripheral blood samples from HS patients, neutrophils had enhanced NETosis and WB analysis revealed that these NETs possessed proteins recognized by autoantibodies (AAbs) present in HS serum, namely antibodies against IL-17B. Furthermore, serum from HS patients had significant titers of total IgG and contained AAbs against citrullinated proteins, including filaggrin, vimentin and enolase corresponding to levels detected in sera from patients with rheumatoid arthritis (p<0.05). Moreover, NETs were confirmed in HS tissue via immunofluorescent detection of citrullinated histone 4 (cit-H4). With ELISA, HS tissue homogenates revealed a positive correlation of detected cit-H3/double-stranded DNA complexes with disease stage (r2=0. 7107, p=0.0043). Finally, HS tissue displayed a significant upregulation of IFN genes. Taken together, these results suggest unreported roles of autoimmunity and neutrophils in the pathogenesis of HS, identifying NETs as a source of AAbs and the type I IFN signature in HS tissue, which could impact alterations in therapeutic approaches.

P37 NEUTROPHIL EXTRACELLULAR TRAPS IN ACUTE BACTERIAL MENINGITIS

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Background: Pneumococcal acute bacterial meningitis (ABM) is a severe condition with very high mortality. During ABM there is a large influx of neutrophils into the cerebrospinal fluid (CSF). Neutrophil extracellular traps (NETs) can be beneficial or detrimental to the host. Whether NETs are formed in human ABM or other brain inflammatory conditions is not known. Our objective was to determine whether NETs are formed in pneumococcal ABM and whether they play a role in bacterial clearance.
Methods and Results: We visualized NETs by immunofluorescence in CSF of patients with pneumococcal ABM, viral meningitis, subarachnoid hemorrhage, and neuroborreliosis and found NETs exclusively in pneumococcal ABM patients. We also detected NETs by this method in the CSF in a rat pneumococcal meningitis model. DNase treatment of infected rats significantly reduced NETs in the CSF and reduced the number viable bacteria both in the brain and in other organs. In vitro, we stimulated isolated human neutrophils with pneumococci and DNase. DNase treatment significantly increased phagocytosis in a gentamicin protection assay, increased myeloperoxidase (MPO) activity and reduced bacterial viability.
Conclusions: The exclusive presence of NETs in human ABM and in a rat pneumococcal meningitis model could indicate a novel diagnostic role for NETs. Removal of NETs by DNase treatment increased bacterial killing and innate neutrophil killing mechanisms and also reduced bacterial dissemination from the brain to the other organs, suggesting that the presence of NETs may not be beneficial and that NETs could be a potential therapeutic target in pneumococcal ABM.

P38 EARLY AND LATE EVENTS OF NETOSIS ARE CONTROLLED BY DISCRETE SIGNALING PATHWAYS

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Neutrophil extracellular traps influence pathogenesis in chronic inflammation, autoimmunity, and cancer. Despite the importance of NETs, the molecular mechanisms underlying their formation, as well as the upstream signaling pathways involved, are only partially understood. Current methodological approaches to quantify NETs also suffer from significant drawbacks. We used novel, fluorescent polymers that only bind extruded chromatin, allowing specific and standardized quantification of NETosis. In cells activated with various physiological stimuli, inhibition of the TAK1, p38 MAPK, or MEK pathways hindered NETosis by acting on early events that influence the length and degree of branching of extruded chromatin filaments, as opposed to chromatin extrusion itself. Evidence suggests that PAD4 activation ranks among such early events. By comparison, inhibiting Syk or P3K nearly abolished NETosis; this involved late signaling events (occurring at about 120 min of stimulation), i.e. chromatin extrusion and/or upstream processes. The nature of the late processes affecting NETosis, however, remains elusive. We could exclude newly-made cytokines and chemokines as potential candidates, since neither cycloheximide nor actinomycin D were found to affect NETosis in response to any of the stimuli used. Finally, inhibiting Src family kinases or JNK failed to prevent NETosis. Our data substantially extends current knowledge of the signaling pathways controlling NETosis, and reveals how they affect early or late stages of the phenomenon. In view of the involvement of NETs in several pathologies, our findings also identify molecular targets that could be exploited for therapeutic intervention.

P39 CHEMOTACTIC, PHAGOCYTIC AND NEUTROPHIL EXTRACELLULAR TRAP (NET) FORMING PROPERTIES OF ORAL AND CIRCULATORY BLOOD NEUTROPHILS

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Oral health maintenance is, in part, managed by the immune-surveillance and antimicrobial functions of PMNs that have migrated from the blood circulation (cPMN) through oral mucosal tissues as oral PMNs (oPMNs). PMNs migrate towards exogenous chemoattractants, phagocytose bacteria and produce NETs to immobilize and eliminate pathogens. We hypothesized that oPMNs offer a relevant model to study the role of PMNs in maintaining oral health. We compared chemotactic, phagocytic and NET formation capacities of oPMNs and cPMNs. oPMNs and cPMNs were isolated from healthy donors. Directional chemotaxis towards the chemoattractant fMLP was analysed using an Insall chamber and video microscopy. Phagocytosis was analysed by flow cytometry based on CD16+FITC+ gating of PMNs incubated with heat-inactivated FITC-labelled Fusobacterium nucleatum (Fn). NET formation by oPMNs and cPMNs was quantified fluorometrically using Sytox Green after stimulation with either PMA or RPMI medium (unstimulated control). In contrast to cPMNs, chemotactic responses of oPMNs towards fMLP did not differ from unstimulated controls. oPMNs show reduced speed, velocity and directional movement towards fMLP when compared to cPMNs, which could be explained by exhausted chemotaxis capacity after having migrated through oral tissues into the oral cavity. oPMNs and bPMNs phagocytosed Fn similarly. Unstimulated and stimulated oPMNs formed significantly more NETs than cPMNs. Based on observed normal phagocytosis but hyperreactive NET production by unstimulated oPMNs, we conclude that oPMNs are primed due to their exposure to oral bacteria.

P40 MORE THAN A BIOMARKER: CRP INDUCES NETOSIS IN HEART FAILURE PATIENTS WITH OR WITHOUT DIABETES

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C reactive protein (CRP) is recognized as a biomarker of chronic, low-grade inflammation associated to vascular disorders. Lately, the role of NETs has been interrogated as a potential source of chronic inflammation and obliteration of small blood vessels in cardiovascular pathologies. The primary objective was to investigate neutrophil extracellular traps (NETs) as a marker of inflammation in patients with heart failure (HF) with or without type 2 diabetes (T2D). The secondary objective was to examine the correlation between NETs and CRP in these patients. This was a non-interventional study including patients with HF±T2D and age-matched healthy volunteers (HC) group. Serum contents of NETs and other inflammatory markers were measured by ELISA. The release of NETs by the neutrophils under various stimuli was measured by confocal microscopy. The levels of NETs in the serum of HF patients were significantly higher as compared to HC (106% increase; $p=0.014$). Neutrophils from HF and HFT2D patients were primed to NETs synthesis as compared to T2D and HC groups ($p\leq0.04$). Serum CRP concentrations were significantly increased in all 3 groups of patients (HF, T2D and HFT2D) as compared to HC ($p\leq0.03$), and a positive correlation was observed between serum CRP and NETs levels ($p=0.03$). Under in vitro condition, CRP induced a concentration-dependent NETs synthesis.This study proposes a mechanism by which CRP increases the risk of future cardiovascular events, and supports mounting evidences on the role of neutrophils in chronic low-grade inflammation associated to heart failure.

P41 HUMAN NEUTROPHIL EXTRACELLULAR TRAPS SYNTHESIS CONTRIBUTES TO ANGIOPOIETIN-MEDIATED IN VITRO PROINFLAMMATORY AND PROANGIOGENIC ACTIVITIES

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Neutrophil extracellular traps (NETs) are composed of nuclear DNA in a web-like structure extruded from neutrophils in response to either bacterial infection or inflammation. We previously reported the expression of angiopoietin Tie2 receptor on human neutrophils and the capacity of both angiopoietins (Ang1 and Ang2) to induce proinflammatory activities, such as synthesis and release of platelet-activating factor, upregulation of b2 integrin complex (CD11/CD18), and neutrophil chemotaxis. In contrast, only Ang1 but not Ang2 is capable of promoting translational and transcriptional activities in neutrophils. In this article, we addressed whether Ang1 and/or Ang2 could modulate the release of NETs and if they contribute to angiopoietin-mediated proinflammatory activities. We observed that Ang1 and Ang2, alone or combined (10 nM, 3 h), increase NET synthesis and release by 2.5-fold as compared with PBS-treated neutrophils. The release of NETs is Tie2 dependent and requires downstream intracellular participation of PI3K, p38, and p42/44 MAPK pathways; reactive oxygen species production; intracellular calcium store depletion; and protein arginine deiminase 4 activation. These isolated NETs induced neutrophil and endothelial cell activation, leading to neutrophil adhesion onto human extracellular matrix and HUVEC and in vitro formation of capillary-like tubes by endothelial cells. To our knowledge, our study is the first one to report the capacity of Ang1 and Ang2 to promote the release of NETs and that these NETs contribute to angiopoietin-mediated in vitro proinflammatory and proangiogenic activities.

P42 THE FLAVONOID GALANGIN INDUCES NET FORMATION, ENHANCES BACTERIAL CLEARANCE AND RESOLUTION OF ACUTE LUNG INJURY

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Impaired neutrophil bactericidal activity and prolonged neutrophil survival contribute to the development of acute respiratory distress syndrome (ARDS) in patients with sepsis, and portend poor prognosis. Galangin induces autophagy in certain cancer cells, which may trigger release of neutrophil extracellular traps (NET). In this study, we investigated the impact of galangin on neutrophil bactericidal activity and apoptosis, critical events in determining the outcome of ARDS. Culture of human neutrophils with galangin did not alter ROS production, surface expression of complement receptors and phagocytosis, whereas it enhanced killing of E. coli from both intra- and extracellular compartments without exerting a direct bactericidal activity. Galangin evoked release of elastase, citrullinated histone and DNA, hallmarks of NETosis. These actions were prevented by DNAse-I, but not by pharmacological blockade of NADPH oxidase. Galangin enhanced mitochondrial ROS production and collapse of mitochondrial transmembrane potential. Galangin also overrode the anti-apoptotic signal from E. coli or E. coli DNA by attenuating phosphorylation of ERK 1/2 and Akt, leading to Mcl-1 degradation. In a mouse model of E. coli-induced acute lung injury, galangin administered at the peak of inflammation, accelerated clearance of bacteria, increased alveolar DNA content and reduced the ratio of pro- and anti-inflammatory cytokines, thereby facilitated inflammation resolution. Collectively, these results identify induction of NETosis as a novel mechanism by which galangin could facilitate bacterial clearance and ultimately the resolution of ARDS.

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P43 CANDIDA AURIS BLOCKS NEUTROPHIL FUNCTION

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Candida auris, an emerging fungal pathogen, causes outbreaks of invasive candidiasis with mortality as high as 60%. Little is known about the pathogenesis of this species that has newly arisen in the last 10 years, and it is unclear why this species is rapidly spreading worldwide. Neutrophils kill fungi through phagocytosis or release of neutrophil extracellular traps (NETs). The objective of this study was to delineate the neutrophil response to C. auris. We hypothesized that neutrophil dysfunction may account for the poor outcomes observed in patients. We examined interactions of human neutrophils with C. auris and included C. albicans for comparison. Interactions were visualized by time-lapse fluorescent microscopy and scanning electron microscopy (SEM). We utilized oxidative stress indicator CM-H2DCFDA to measure the generation of reactive oxygen species (ROS) in neutrophils. NET formation was quantified by Sytox Green staining and assessed by SEM and immunofluorescent labeling of NET-associated proteins. Fungal viability was evaluated using Live-or-Dye viability staining and plate counts. C. auris triggered minimal NET release by neutrophils, with levels 7-fold lower when compared to C. albicans. Similarly, SEM revealed extensive NET formation in response to C. albicans, but not C. auris. The generation of ROS, a key signaling mechanism for NET formation, was also dampened in neutrophils encountering C. auris. The ineffective neutrophil response to C. auris correlated with diminished fungal killing. In conclusion, we identified impaired release of NETs in response to C. auris. This is linked to improved fungal survival. We propose that this altered innate immune response may contribute to the unexpected virulence of C. auris by allowing this species to evade killing by neutrophils.

P44 QUANTIFICATION OF NEUTROPHIL EXTRACELLULAR TRAP FORMATION USING AN ELISA SPECIFIC FOR CITRULLINATED HISTONE H3.

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The objective of this study was to develop an ELISA specific for citrullinated histone H3 that could be used to accurately and reproducibly quantify NETs from multiple species. A monoclonal antibody (11D3) was produced against the tail of histone H3 citrullinated at arginines 2, 8, and 17). A second monoclonal antibody (2D6) that is specific for a highly-conserved epitope on unmodified histone H3 was isolated from a mouse (NZB/W F1) with spontaneous lupus. These antibodies form a sandwich pair that recognizes citrullinated histone H3 that is released during the process of NET formation. PAD4-citrullinated HeLa core histone H3 is used as a quantifiable standard in the ELISA. The Citrullinated Histone H3 ELISA can detect and quantify CitH3 from NETs produced by human peripheral blood neutrophils and mouse bone marrow neutrophils after treatment of the NETs with S7 nuclease to release the DNA-bound CitH3. The ELISA also detects CitH3 from the plasma of LPS challenged mice. CitH3 can be detected in the detergent-insoluble fraction of neutrophil lysates early after neutrophil stimulation, but prior to mature NET formation. The detection and relative quantification of NET formation has relied primarily upon visualization by fluorescence microscopy which is not accurately quantifiable, or measurement of extracellular DNA which is quantifiable, but not exclusive to NET formation. Attempts have been made to quantify NET formation by using heterobifunctional ELISAs detecting various combinations of myeloperoxidase, neutrophil elastase, and DNA, but these suffer from a lack of reliable, reproducible standards. The CitH3 ELISA provides a quantitative, reproducible, selective, and inexpensive method for assessing NET formation.

P45 LOCAL DELIVERY OF CL-AMIDINE FROM ELECTROSPUN TEMPLATES REGULATES ACUTE NEUTROPHIL NETOSIS AND BIOMATERIAL PRECONDITIONING

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During acute inflammation, swarming neutrophils can release NETs on the surface of implanted biomaterials as a potentially significant preconditioning event for biomaterial-guided tissue regeneration. In this study, we used electrospun polydioxanone (PDO) templates as a delivery vehicle for Cl-amidine to modulate PAD4-mediated, template-induced NETosis. Small fiber diameter (0.2-0.4 microns) and large fiber diameter (1.0-3.0 microns) PDO templates were electrospun from polymer solutions with 0-5 mg/mL Cl-amidine. Acute neutrophil-template interactions were evaluated in vitro with human neutrophils and in vivo with a 24-hour rat subcutaneous implant model. The in vitro results suggest that significant dose-dependent inhibition of PAD4-mediated NETosis occurs on the small diameter templates while the opposite is observed on large diameter templates, indicating multiple pathways regulate biomaterial-induced NET release. Similar results were observed in vivo for the small diameter templates and verify that electrospun PDO templates function as a delivery vehicle for Cl-amidine to inhibit acute, local NETosis in a physiological environment. Furthermore, the large diameter templates with Cl-amidine enhanced neutrophil invasion and survival in vivo compared to the small diameter templates, which indicates the potential for long-term modulation of tissue regeneration by the neutrophil. Taken together, this study utilizes a novel delivery vehicle for Cl-amidine to regulate biomaterial-induced NETosis and demonstrates the need to explore neutrophil interactions in biomaterial-guided tissue regeneration.

P46 PROTEOLYTIC MODIFICATION OF NET COMPONENTS AFFECTS THEIR RECOGNITION BY AUTOANTIBODIES

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Neutrophils are able to eject their nuclear material in the extracellular space to form a Neutrophil Extracellular Trap (NET) in a process called NETosis. NETs can capture pathogens, but when not properly cleared, autoimmune responses might be elicited to NET components. Neutrophil proteases play an essential role in NET formation, but they also affect the protein content of the NETs. For example, the histones seem to be prone to degradation by proteases. We show here that the serine protease inhibitor PMSF is able to prevent histone cleavage in the NETs. Histone 3 citrullination, a molecular hallmark of NETs, was detected on PMSF-treated NET harvests but not on non-treated NETs. The conversion of NET-associated proteins by the neutrophil proteases might generate neo-epitopes of autoantibodies. Alternatively, the cleavage of NET-associated autoantigenic proteins may result in the loss of autoepitopes. Preliminary data suggest that RA patient sera, which frequently contain autoantibodies to citrullinated proteins, including citrullinated histones, show more reactivity with NETs produced in the presence of PMSF than with non-treated NETs. Our data are consistent with an important role for neutrophil protease activities in the recognition of NET components by autoantibodies.

P47 DEVELOPMENT OF AN ANTIDOTE FOR EXTRACELLULAR HISTONE-MEDIATED PATHOLOGIES

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Neutrophils, following activation, extrude their chromatin as complex extracellular networks, termed neutrophil extracellular traps (NETs), that can capture and kill bacteria. NETs, however, also have pathological effects, particularly via their highly cationic histones that can be NET associated or released from NETs by nucleases. In fact, free histones are cytotoxic for endothelial cells, initiate coagulation by activating platelets and induce anaemia by enhancing erythrocyte fragility. Such pathological effects resemble those seen in sepsis and, indeed, it has been shown that extracellular histones are the major cause of death in sepsis. Thus we reasoned that small polyanionic molecules should interact electrostatically with the highly cationic histones and neutralise their pathogenic effects. Initially, a small library (~50) of polyanions (PAs) was screened for molecules that have such activity and several small PAs (~1 kDa) identified that in vitro simultaneously inhibited the cytotoxic, platelet activating and erythrocyte damaging effects of histones. Remarkably, histone damaged endothelial cells and erythrocytes also could be rapidly rescued by addition of the PAs hours after initial histone exposure. Subsequent in vivo studies showed that the PAs could totally inhibit histone-induced sepsis in mice, namely thrombocytopenia, leukopenia, anaemia and multi-organ failure. The small PAs also prevented death and multi-organ damage in a rat caecal ligation puncture model of sepsis but did not inhibit the early cytokine storm phase of the disease. Thus, we have identified a new class of drugs that protects against multi-organ failure in sepsis by binding electrostatically to histones and acting as an extracellular histone antidote.

P48 NEUTROPHIL EXTRACELLULAR TRAPS IN ASTHMA: DIFFERENT IMPACTS DEPENDING ON THEIR LOCALIZATION

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Introduction

Neutrophil extracellular traps (NETs) are web-like structures formed during neutrophil activation. To date, several lines of evidence suggest a major role for NETs in a broad range of human diseases but their involvement in asthma remains poorly understood. To investigate the presence of NETs in a large cohort of asthmatic adult patients and their relationship with inflammatory phenotype and clinical features of asthma. Circulating NETs were evaluated in 218 asthmatic patients from the COBRA national cohort, using DNA-DNA-MPO ELISA (myeloperoxydase). Local production of these extracellular traps was assessed by evaluating this marker in 52 BAL (bronchoalveolar) supernatants. High circulating NET levels were associated with poor asthma control and low reversible bronchial obstruction. In contrast, when analyzing local production of NETs, we found that BAL NETs levels were significantly lower in severe asthma as compared with moderate asthma. Local NETs levels were associated with bronchial neutrophilia. We found higher BAL NETs levels when patients had a subclinical pulmonary infection documented by positive culture in bronchial aspirate. Taking together, these results suggest a different impact of NETs in asthma according to their localization. Systemic production is associated with poor asthma control, whereas alveolar production seems to be related to less severe phenotype, probably in relation with their anti-infectious properties.

P49 ADRENOMEDULLIN REDUCES NEUTROPHIL EXTRACELLULAR TRAP FORMATION AMELIORATING LUNG INJURY IN SEVERE PNEUMOCOCCAL PNEUMONIA

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Pneumococcal pneumonia remains a significant health problem worldwide. Streptococcus pneumoniae (S.pn.) promotes dysregulation of the immune system and disruption of the endo-epithelial barrier leading to acute lung injury and sepsis. Neutrophil extracellular traps (NETs) are produced upon infection and have a harmful role in acute lung injury. We showed previously that the hormone-peptide adrenomedullin (ADM) is protective against S.pn.-induced lung barrier disruption, edema formation and extra-pulmonary organ damage. Here, we hypothesize that ADM exerts its protective functions also by targeting NET formation.

In a murine model of severe pneumonia, mice were infected with S.pn. (5x10^6 CFU, intranasal) and then treated with ADM or DNase. The mechanistic properties of ADM were assessed in vitro in human blood neutrophils by analyzing NET formation, ERK activation, and ROS generation. Neutrophils accumulate into the lungs after S.pn. infection and release excessive amounts of NETs, which may contribute to systemic inflammation and tissue damage. NET degradation with DNase attenuates S.pn.-induced lung permeability, suggesting a harmful role of NETs in pneumonia. ADM treatment led to a reduction of neutrophil activation and NET release. In vitro, ADM suppressed NET production from stimulated neutrophils through a mechanism that involves increase of cAMP production, inhibition of ERK phosphorylation and decrease in ROS production, necessary for NET formation.

Our results indicate that ADM reduces lung injury and extra-pulmonary organ failure by suppressing NET release and it could be considered a promising therapeutic strategy in severe pneumonia.

P50 ENDOGENOUS REGULATION OF NEUTROPHIL EXTRACELLULAR TRAPS

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Objective: There is increasing evidence of the pathophysiologic role of neutrophil extracellular traps (NETs), however little is known of their endogenous regulation. Adenosine (ADO) has anti-inflammatory effects on PMNs. Similarly, mesenchymal stromal cells (MSCs) are an anti-inflammatory cell therapy used to treat hyper-inflammatory diseases. MSCs can produce extracellular ADO via surface ectonucleotidases (CD39, CD73). Thus we studied the effects of MSCs and ADO on NETs.

Methods: PMNs were isolated from blood of healthy donors and induced to make NETs using phorbol 12-myristate 13-acetate (PMA). PMNs were treated with ADO and ADO receptor agonists and antagonists. Bone marrow derived MSCs were cultured under standard conditions and treated with the CD73 antagonist alpha, beta-methyleneADP (APCP). NET formation was quantified as the relative inhibition percent compared to the same PMNs treated with only PMA. NETs were quantified by Sytox green assay and confirmed with elastase-DNA ELISA.

Results: ADO, and the non-selective ADO receptor agonist NECA, reduced NET formation (ADO 28.8± 7.5%; NECA 30±11.9%, p<0.05, n=7). A2A and A2B adenosine receptor agonist reduced NETs to a similar degree (CGS15943, 29.6±9.9%; BAY 606583, 29.3±6.9%, n=10). Only the A2A specific antagonist abrogated the anti-NET effects of adenosine (ADO+ZM241385, 13.8±10.7%, n=6). Co-culture of PMNs and MSCs reduced PMA induced NETs by 35±22.5% (n=18, p<0.05), but not when MSCs were pretreated with APCP (18.4±25.6%, n=7). APCP treated MSCs were also unable to produce extracellular ADO in AMP supplemented media.

Conclusion: ADO, signaling through the A2A receptor, may be an important endogenous regulatory mechanism of netosis. Extracellular ADO produced by MSCs via CD73 regulates NETs and may be critical for the anti-inflammatory effects of MSCs.

P51 CLEC5A IS CRITICAL FOR NET FORMATION AND HOST DEFENSE AGAINST BACTERIAL INFECTION.

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Neutrophil extracellular traps (NETs) play critical roles in host defense against microbial invasion. Here we show that CLEC5A is critical for bacteria-induced NET formation, and is responsible for histone citrullination and nuclear translocation of elastase. Severe impairment of NET formation correlated with extensive bacteria spreading in liver and bacteremia at 4 hour after infection, and Clec5a-/- mice were highly susceptible to bacterial infection. Interestingly, CLEC5A and TLR2 were upregulated and co-localized after incubation with bacteria, and co-activation of CLEC5A and TLR2 further enhanced NET formation via upregulation of free radical production and activation of p38 MAPK and AKT pathways. More severe liver necrosis and bacteremia were observed in Clec5a-/-Tlr2-/- mice than Clec5a-/- mice, and all Clec5a-/-Tlr2-/- mice died at day 5 post inoculation of sublethal dose of L. monocytogenes. Thus, CLEC5A is not only critical for NET formation, but also collaborates with TLR2 in host defense against systemic bacterial infection.

P52 NEUTROPHIL EXTRACELLULAR TRAPS ARE INDUCED BY ADENOSINE AND STIMULATE RELEASE OF TNF-ALPHA FROM MACROPHAGES IN DEFICIENCY OF ADENOSINE DEAMINASE 2 (DADA2)

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Reduction of adenosine deaminase 2 (ADA2) activity due to autosomal recessive loss of function mutations in the CERC1 gene results in a systemic illness known as deficiency of ADA2 (DADA2), characterized in part by early-onset stroke, small-medium vessel vasculitis, and clinical response to TNF-inhibitors. Neutrophils and a subset of neutrophils known as low-density granulocytes (LDGs) have been implicated in the pathogenesis of vasculitis through the formation of neutrophil extracellular traps (NETs). The study objective was to determine whether neutrophils and NETs play a pathogenic role in DADA2. In vivo evidence demonstrated NETs and macrophages in affected gastrointestinal tissue in a patient with DADA2. An abundance of circulating LDGs were observed during active disease in DADA2 and were significantly reduced after remission induction by anti-TNF therapies. Adenosine triggered NET formation by engaging A1 and A3 adenosine receptors (ARs) and through ROS- and PAD4- dependent pathways. Adenosine-induced NETosis was inhibited in the presence of recombinant ADA2, A1/A3 AR antagonist, or an A2A agonist. M1 macrophages incubated with NETs from patients with DADA2 released significant amounts of TNF-alpha. Treatment with an IL-1 receptor antagonist (anakinra) or an A2A AR agonist decreased nuclear translocation of NFkappaB and subsequent production of inflammatory cytokines in macrophages. These results suggest that neutrophils may play a pathogenic role in DADA2. Modulation of adenosine-mediated NET formation may contribute a novel and directed therapeutic approach in the treatment of DADA2.

P53 TYPE I AND III IFNs CONTROL THE ANTI-VIRAL RESPONSE IN THE LUNG THROUGH DISTINCT ACTIONS ON NEUTROPHILS

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Lambda interferons (IFN-lambdas) or type III IFNs share homology, expression patterns, signaling cascades, and antiviral functions with type I IFNs. Assigning to them unique, non-redundant roles has therefore been difficult. The aim of this study was to decipher the role of IFN-lambdas during respiratory viral infection. We used an established mouse-adapted strain of H1N1 Influenza virus (strain A/PR/8/34) (IAV) to infect mice unresponsive to either IFN-lambdas or type I IFNs or both. We analyzed several disease parameters including weight loss, morbidity and lung function, inflammatory cell infiltrates and cytokine concentrations in the bronchoalveolar lavage fluid (BAL), viral load in BAL and total lung tissue, and performed histological analysis. Moreover, we analyzed the transcriptomic profile of IFN-lambdas and type I IFNs. We found that IFN-lambdas are the first and predominant IFNs produced in the lung upon IAV infection mediating antiviral protection without activating inflammation. Type I IFNs come into play later during infection to enhance viral resistance, but at the same time they induce pro-inflammatory responses essential for confronting infection but also causing immunopathology. Central to this are neutrophils, which respond to both types of IFNs to upregulate antimicrobial functions but exhibit pro-inflammatory activation only to type I IFNs. Ongoing studies in neutrophils from healthy individuals and patients with viral pneumonia also support an important antiviral role of IFN-lambdas in respiratory infections. Conclusively, IFN-lambdas constitute the front line of antiviral defense in the lung without compromising host fitness.

P54 PROTEOMIC ANALYSIS OF NEUTROPHILS FROM PROTEINASE 3 ANCA-ASSOCIATED VASCULITIS REVEALS DISTURBED APOPTOTIC PATHWAYS AND A DYSREGULATION IN MEMBRANE PROTEINS

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Neutrophils are the target of autoimmunity in vasculitis characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA) and are key effector cells responsible for endothelial damage. In granulomatosis with polyangiitis (GPA) proteinase 3 (PR3) is the target of ANCA present in more than 95% patients. Apoptotic neutrophils from those patients have increased membrane PR3 which is associated with proteins involved in recognition and clearance of apoptotic cells. Based on the hypothesis that there is a disturbance in both apoptosis and immune mechanisms associated with apoptotic cell clearance, we performed a proteomic analysis of the cytosol of neutrophils from GPA patients compared to healthy controls (HC) cells. At disease onset, the cytosolic proteome of GPA neutrophils before treatment was significantly different from HC and this dysregulation was more pronounced following ex-vivo induction of apoptosis. Expression of proteins involved in cell death/survival was altered in GPA neutrophils and a number of hits were known PR3-binding partners involved in the clearance of apoptotic cells, namely calreticulin, Annexin A1 and phospholipid scramblase 1. Our study provides evidence that GPA neutrophils have an intrinsic dysregulation in proteins involved in apoptosis and mechanisms of their clearance which likely contribute to a defect in the resolution of inflammation. Harnessing these dysregulated pathways could lead to novel biomarkers and targeted therapeutic opportunities.

P55 REVEALING THE NEUTROPHIL GRANULE-SPECIFIC N-GLYCAN SIGNATURE

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Protein glycosylation is implicated in multiple aspects of innate immune response and regulation. Aberrant glycosylation can weaken the immune response, including the function of neutrophils. Apart from our recent characterization of paucimannosidic N-glycans on intact proteins within azurophil granules (AG), neutrophil glycan data are based primarily on whole-cell preparations. Thus, a detailed structural map of neutrophil granule glycosylation remains to be established. Human neutrophils were isolated from buffy coats from healthy donors and AG, specific/gelatinase granules (SG/GG), as well as secretory vesicles/plasma membrane (SV/PM) fractions were separated by Percoll density gradient centrifugation. The granular proteins were extracted, N-glycans were enzymatically released using PNGase F and analyzed on mass spectrometry (MS). Distinct N-glycans were characterized across neutrophil granules, with significant granule-specific glycan differences. Paucimannosidic N-glycans were remarkably abundant in the AG. The profile of SG/GG was different with complex and very long bi-antennary N-glycans. MS/MS analysis of these long glycans of high m/z confirmed the presence of elongated poly-LacNAc structures (up to 17 repeats) with and without fucose residues and capped with sialic acid in the SG/GG. SV/PM were characterized by high mannose glycans and poly-LacNAc structures. This first glycomic mapping of neutrophil granule glycans highlights novel granule-specific glycans and is a necessary first step to a detailed glycoproteomics map of granule proteins. This work will provide a platform to identify unusual protein glycosylation, unique to distinct human neutrophil populations in disease.

P56 COMPLEMENT-INDUCED NEUTROPHIL DYSFUNCTION: INSIGHTS FROM WHOLE BLOOD FUNCTIONAL ASSAYS AND PHOSPHOPROTEOMICS

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Susceptibility of critically ill patients to nosocomial infection is strongly associated with immune-cell failure; however the mechanisms underpinning this process remain incompletely understood. Previously, we have demonstrated that the anaphylatoxin C5a impairs phagocytosis by healthy donor and patient neutrophils. Here we investigated signaling mechanisms driving neutrophil dysfunction in this context. Phagocytosis in human whole blood was quantified by flow cytometry using an Attune Acoustic Focusing Cytometer (Life Technologies). Complete proteomes and phosphoproteomes of phagocytosing neutrophils were obtained from 4 healthy donors pre-treated with C5a or vehicle. C5a rapidly reduced phagocytosis of S. aureus in whole blood by 39.5 % ($p = 0.003$). Moreover, this phagocytic impairment increased over time following C5a exposure. In contrast to C5a, LPS and PAF increased phagocytosis ($p = 0.008$ and $p = 0.022$ respectively). Quantitative proteomic and phosphoproteomic analysis allowed quantification of 4859 and 2712 proteins, respectively. Significant alterations in protein phosphorylation patterns revealed possible novel pathways involved in complement-induced neutrophil dysfunction. This is the first study to demonstrate the selective ability of C5a to impair neutrophil phagocytosis of a clinically relevant pathogen in a whole blood model, which mimics bacteraemia. Quantitative phosphoproteomic profiling to this depth provides unprecedented insights into key neutrophil functions of relevance to critically ill patients.

P57 METABOLOMIC ANALYSIS OF HUMAN NEUTROPHILS

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Transcriptomic analysis of neutrophils has revealed significant differences in gene expression during inflammation. However metabolomics analysis of healthy or inflammatory neutrophils has not been described. The aim of this study was to establish protocols for the study of human neutrophils using 1H NMR metabolomics.

Sample preparation and spectral analysis protocols were optimised using healthy neutrophils, including a heat-shock step to prevent metabolite loss. Hepes-free media was used to incubate cells ±PMA for 15 min. Triplicate samples were analysed using a 700 MHz NMR Avance IIIHD Bruker NMR spectrometer equipped with a TCI cryoprobe. Chenomx, Bruker TopSpin and AMIX software were used to identify metabolites and process spectra. Spectra were analysed using ChenomxP and Bruker TopSpin software to identify metabolites. Statistical and signalling pathway analysis was carried out using Metaboanalyst.

Cell number and number of scans (NS) was optimised to >3.5 million cells and 512 NS. 327 spectral bins were identified, of which 287 (87.7%) were assigned to 110 metabolites including: amino acids and peptides; carbohydrates, carbonyls and alcohols; nucleotides and nucleosides; lipids and lipid-like molecules; benzenoids; and other organic compounds. 43 metabolites changed >1.5 fold (increase/decrease) with the addition of PMA. Pathway analysis revealed PMA affected nicotinate and nicotinamide metabolism, aminoacyl-tRNA biosynthesis and glycolysis, suggesting a redirection of glucose metabolism from glycolysis to the pentose phosphate pathway and production of NADPH for activation of the NADPH oxidase and subsequent respiratory burst.

Our methodology is a sensitive enough to detect changes in metabolite abundance from cell counts typically collected from clinical samples or experiments with multiple assay conditions.

P58 INTESTINAL ISCHEMIA AND REPERFUSION MODULATES THE RAT NEUTROPHILS PROTEOME

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Objective: Intestinal ischemia and reperfusion often results into systemic inflammatory response and organ failure with the participation of neutrophils. However, the molecular mechanisms by which ischemia and reperfusion stimulate circulating neutrophils is still unclear. In this work we used proteomic analysis to evaluate the effect of ischemia and reperfusion on neutrophils in Wistar rats. Methods: We isolated neutrophils from three groups: control, sham laparotomy, and intestinal ischemia/reperfusion. Neutrophil proteins from each group were iTRAQ-labeled and submitted to LC-MS/MS analysis. Results: Our proteomic analysis revealed 426 proteins as significantly regulated in at least one of the analyzed conditions. Interestingly, the enzyme prediction analysis revealed that ischemia reperfusion significantly reduced the abundance of most of the antioxidant and pro-survival molecules to cause more tissues damage and ROS production whereas some of the significantly up regulated enzymes were involved in cytoskeletal rearrangement, adhesion and migration. Cluster based KEGG pathway analysis revealed the high motility, phagocytosis, directional migration, and activation of the cytoskeletal machinery in neutrophils following the ischemia and reperfusion. Increased ROS production and decreased phagocytosis were experimentally validated by microscopy assays. Conclusion: Taken together, our findings provide a characterization of the effects of intestinal ischemia and reperfusion on neutrophil proteome and the possible mechanisms involved in the tissue injury by neutrophils after intestinal ischemia and reperfusion.

P59 MORPHOLOGY CHANGES AND REACTIVE OXYGEN SPECIES GENERATION BY NEUTROPHIL INTERACTION WITH TIO2 PARTICLES.

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Objective: The growing use of TiO2 is a worrying factor due to the long-term risk of toxicity. This study aimed to investigate the capture of TiO2 fine particles (FPs) and nanoparticles (NPs) aggregates by neutrophils and analyze their effect on the cell viability, morphology, ROS production, phagocytosis and adhesion molecules. Methods: Neutrophils were exposed to TiO2 FPs and NPs for 1, 5, 30 and 60 minutes. The cell morphology was assessed by Light Microscopy (LM), Scanning and Transmission Electron Microscopy (SEM, TEM). Membrane adhesion molecules were analyzed by flow cytometry. The NBT test enabled evaluation of cell activation through the detection of superoxide anion. Videomicroscopy (VM) evaluated neutrophils displacements and phagocytosis of TiO2 FPs and NPs aggregates. Results: VM, LM and SEM results showed that the time factor had a significant effect on PMN phagocytosis and morphological changes. The expression of tubulovesicular extensions was observed after 30 minutes of incubation, as well as NETs. The NBT test shows a significant effect of the exposure to the particles and of the exposure time on neutrophil superoxide production. The surface marking of human neutrophils with anti-CD11b and anti-CD62L demonstrated no statistically significant effect of TiO2 FPs and NPs. Conclusion: Interaction of neutrophils with TiO2 FPs and NPsaggregates promoted time dependent decreased cell viability while increased ROS production, formation of tubulovesicular extensions, vesicles, pores and NETs without significantly changing CD62L and CD11b levels.

P60 HIGH PERFORMANCE MASS SPECTROMETRY BASED PROTEOMICS OF NEUTROPHILS EXPOSED TO TIO2 PARTICULATES

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Objective: The use of TIO2 is widely distributed in clinical practice and is considered by many to be inert to the body, however particles of this molecule are toxic to different cell types and have proinflammatory properties. This study aimed to investigate changes in the protein profile and biological processes induced by the interaction of fine particles (FPs) and nanoparticles (NPs) of TiO2 with neutrophils. Methods: Neutrophils were exposed to TiO2 particles for 30 minutes (an aliquot was kept in buffer for control). The cell morphology and viability were analyzed by light microscopy and the cellular activation was evaluated by the NBT test, through the detection of superoxide anions. The total protein extracts were digested, the tryptic peptides were submitted to reversed-phase chromatography coupled to high-resolution mass spectrometry. Results: Of the proteins identified, 111 were differentially regulated by exposure to TiO2. When analyzing the functional pathways associated to the regulated proteins, catabolism, endocytosis, phagosome formation and regulation of actin cytoskeleton were found significantly regulated indicating the influence of TiO2 phagocytosis in adhesion, transmigration processes, what is in agreement with another study from our group . Conclusion: The interaction of neutrophils with TIO2 particles has influenced the abundance of proteins involved in catabolism and cell motility pathways, as well as proteins directly involved in neutrophil degranulation and adhesion. Such results represent a starting point to evaluate the influence of biomaterials in the molecular response of neutrophils.

P61 GOOD COPS GONE BAD: NON-CLASSICAL NEUTROPHILS DURING NEISSERIA GONORRHOEAE INFECTION

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The human-specific pathogen Neisseria gonorrhoeae (Ngo) causes the sexually-transmitted disease gonorrhea, an oppressive public health burden due to the recent emergence of multidrug-resistant 'superbug' strains, and the ability of untreated infections to cause inflammation-induced scarring of the reproductive tract. The hallmark of gonococcal infection is a massive influx of neutrophils that do not seem to clear the infection. Primary human and murine neutrophil-based studies demonstrate that opsonin-independent binding and engulfment of Ngo is driven by the bacterial Opa protein adhesin binding to CEACAM (or CD66) expressed on the neutrophil surface. The growing awareness that multiple neutrophil phenotypes exist prompted our consideration that these neutrophil populations may differentially respond to Ngo. If true, this may provide a fundamental shift in our understanding of gonococcal immunity and the immunopathogenesis of infection. The specific objective of this work is to compare Ngo interactions with classical and non-classical neutrophil populations, and the specific response of each neutrophil phenotype to this infection. Our studies to date reveal substantial differences in the neutrophil phenotypes recovered by various purification methods. We observed that classical and non-classical neutrophils both interact with Ngo, but that the well-described neutrophil inflammatory response to Ngo is not apparent in non-classical neutrophil subsets. My ongoing studies aim to compare neutrophil and bacterial factors that mediate microbicidal and immune-regulatory functions of classical and non-classical neutrophil populations. If we can exploit these understandings to develop strategies to stimulate a neutrophil response that will clear infection, then then we can confer long lasting protection against gonococci.

P62 WITHDRAWN

P63 IMPAIRED NEUTROPHIL OPSONO-PHAGOCYTOSIS OF HAEMOPHILUS INFLUENZAE IN COPD AND ALPHA-1 ANTI-TRYPSIN DEFICIENCY

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INTRODUCTION: Alpha-1 Anti-Trypsin Deficiency (AATD) is the most established genetic risk factor for the development of Chronic Obstructive Pulmonary Disease (COPD). Airway colonisation is common in AATD and non-AATD COPD, despite an abundance of neutrophils (PMN) in lung secretions. There is evidence that non-typeable Haemophilus influenzae (ntHI) may induce more inflammation and a faster decline in lung function than other bacteria. Phagocytosis can occur through direct interaction or when bacteria are coated with opsonins. We aimed to determine if impaired opsonisation of ntHI may impair bacterial clearance by PMN in AATD and COPD.

METHODS: Phagocytosis of ntHI and Streptococcus pneumoniae (SP) by isolated blood PMN from n=20 AATD, COPD and age matched healthy controls (HC) was assessed by flow cytometry. Bacteria were not-opsonised or opsonised with pooled AATD, COPD or health age matched control (HC) serum.

RESULTS: PMN phagocytosis of non-opsonised bacteria was not impaired in AATD and COPD vs HC. For all bacteria, opsonisation with pooled HC serum increased phagocytosis vs non-opsonised (p<0.05). PMN phagocytosis of ntHI opsonised with AATD or COPD serum was significantly impaired vs ntHI opsonised with HC serum (MFI±SEM. HC 107870±17379 vs AATD 8509±1164 vs COPD 3656±474, p<0.05). This defect was not ubiquitous but seemed to affect a subset of AATD/COPD patients only. A similar defect was not observed with SP.

CONCLUSIONS: AATD and COPD PMNs can carry out effective phagocytosis of bacteria. However, serum-mediated opsono-phagocytosis of ntHI was significantly impaired in some AATD and COPD patients, compared to health. Understanding this mechanism may offer novel therapeutic targets to reduce bacterial colonisation and clinical decline.

P64 DICHOTOMOUS REGULATION OF MEMBRANE POTENTIAL MODULATES CALCIUM SIGNALING AND DEFINES DISTINCT NEUTROPHIL SUBSETS

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Calcium signals initiated via store-operated calcium entry (SOCE) are critical for neutrophil activation however little is known about the mechanisms that modulate the intensity of these signals. We have observed that bone marrow and splenic neutrophils display two distinct calcium signatures during SOCE, low and high calcium. In exploring why this might be, we discovered that neutrophils display a similar dichotomy in membrane potential during stimulation. Similar proportions of cells segregated into high and low subsets of both membrane potential and calcium (~50% in each), suggesting that these populations are related. Although influx of positively charged calcium ions could cause cell depolarization, co-labeling experiments revealed that neutrophils in the depolarized subset corresponded with calcium-low cells, and cells that maintained polarization had enhanced calcium influx. RNAseq analysis demonstrated that the subsets display distinct transcriptional profiles with differential expression of ~700 genes, suggesting that these are indeed unique populations. Intriguingly, the voltage gated proton channel (Hvcn1), mitochondrial calcium uniporter (MCU/MICU), and voltage gated chloride channel (Clcn3) were all downregulated in the depolarized subset, suggesting that these channels might facilitate maintenance of membrane potential during cell stimulation. Together these results demonstrate that regulation of membrane potential is a major modulator of SOCE in neutrophils and is a defining feature of distinct neutrophil subsets. Studies are ongoing to understand the mechanisms of regulation and interdependence of membrane potential and SOCE in neutrophils, and to further characterize these neutrophil subsets and explore their physiological function.

P65 COMPUTATIONAL APPROACHES TO NEUTROPHIL TRANSCRIPTOME ANALYSIS

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Neutrophils contribute to disease pathology in inflammatory diseases including rheumatoid arthritis (RA), lupus and vasculitis. Using RNA-seq we have (a) replicated the complex RA gene expression profiles observed in vivo by using combinations of agonists in vitro; (b) identified RA blood neutrophil gene biomarkers that stratify RA patients into responders and non-responders to TNFi therapy; (c) characterised a subset of neutrophils in RA (low-density granulocytes, LDGs), which may be novel disease regulators arising from developmental plasticity. This aim of this work is to use computational approaches to construct a model of gene expression in RA which can predict regulators of inflammation in sub-groups of patients (disease responders/non-responders; early/severe disease) and correlate gene expression profiles with clinical demographics and markers of disease activity.

RNA-seq datasets from RA neutrophils (n=15, early RA; n=23 severe RA) were mapped to the human genome (hg38) using Tophat and annotated using Cufflinks. Computational analysis was carried out using ARACNE2 and GALGO. Gene networks were reconstructed using Cytoscape, and functional annotation was carried out using Ingenuity and DAVID. ARACNE2 identified the main co-regulated networks of genes in RA neutrophils as: regulation of transcription, regulation of translation and post-translational modification of proteins, protein transport and localisation, response to activation of an immune cell receptor, innate immune response to interferon alpha. GALGO identified networks of genes which predict clinical characteristics, including disease activity score (DAS28), ESR and CRP titres, and which predict response to TNF inhibitor therapy (measured as a decrease in DAS28). The model will continue to be developed, refined and tested throughout the duration of this project.

P66 USING REAL-TIME DEFORMABILITY CYTOMETRY (RT-DC) TO EXPLORE THE BIOPHYSICAL PROPERTIES OF NEUTROPHILS

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RT-DC is a recently developed, rapid and sensitive way to assess the mechanical properties of cells in suspension (Otto O et al., Nature Methods, 2015). In brief, cells are deformed within a microfluidic constriction channel; simultaneously, a high-speed camera captures images and a cell-tracing algorithm is applied. Off-line analysis generates a “mechanical phenotype” describing cell size, shape and stiffness. Here, we use RT-DC to explore the intersection of neutrophil mechanical properties and function. Patients with active inflammatory disease have a neutrophil mechanical phenotype distinct from healthy controls. Low density granulocytes (LDG), a subset of neutrophils identified in patients with systemic lupus erythematosus (SLE), differ mechanically from neutrophils of healthy individuals. Of note, neutrophils from healthy volunteers also become significantly less dense and larger as they recover from a primed/activated state. However, these ‘induced-LDGs’ clearly differ mechanically from SLE LDGs and are unlikely to be equivalent. Of additional interest, the scientific literature is unclear on whether neutrophils become smaller or larger following activation or priming. RT-DC is uniquely qualified to describe the kinetics of cellular mechanical change. We reveal that following activation or priming, neutrophils undergo a short period of cell shrinking, followed by the cell expansion seen above. In some contexts, neutrophils ultimately recover their un-primed mechanical phenotype. The mechanism(s) underlying these dramatic changes in neutrophil size and deformability are under investigation and would be predicted to have profound implications for cell movement through the vascular system in health and disease.

P67 DIFFERENTIAL CHEMOKINE GRADIENT RESTRICTS NEUTROPHIL RECRUITMENT IN INFLAMED INTESTINES TO THE SUBMUCOSA VASCULATURE

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Neutrophil (PMN) infiltration of the intestinal mucosa is a hallmark of gastrointestinal inflammation, with significant implications for host defense, injury and repair. However, phenotypic and mechanistic aspects of PMN recruitment in inflamed intestines have not been explored in vivo. Using novel epithelial/PMN fluorescence reporter mice, advanced intravital imaging and 3D reconstruction analysis we mapped the microvasculature architecture across the intestinal layers and determined that in response to Salmonella/endotoxin-induced inflammation, PMN transendothelial migration (TEM) was restricted to submucosal vessels. PMN TEM was not observed in villus or crypt vessels, proximal to the epithelium that underlies the intestinal lumen, and was driven specifically in the submucosa by increased chemokine (C-X-C motif) ligands 1 (CXCL1) and 2 (CXCL2) expression. Given the emerging concept of heterogeneity in PMN subsets and function in tissues, chemokine-driven interstitial migration may serve as a novel regulatory step of PMN effector function in inflamed intestines and could be specifically targeted for therapeutic purposes.

P68 IMMATURE NEUTROPHIL DEATH REGULATION BY INTRAVENOUS IMMUNOGLOBULIN (IVIG) IN KAWASAKI DISEASE (KD)

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Band neutrophils are bone marrow-resident precursors of mature, polysegmented neutrophils only found in small numbers in the circulation of healthy individuals. To date, not much is known about cell death regulation in immature neutrophils. In our project, we are investigating the role of immature neutrophils in Kawasaki disease (KD). This self-limiting systemic vasculitis of infants and children is the most common cause of acquired heart disease in Asia and Western countries. The inflammation responds to treatment with high-dose intravenous immunoglobulin (IVIg). However, approximately 15% of patients are resistant to IVIg therapy and have persistent fever following treatment associated with an elevated band count in the peripheral blood. Previous data demonstrate that IVIg triggers cell death in mature neutrophils. We postulate that band neutrophils are resistant to IVIg-mediated cell death and persistence of these cells in the periphery contributes to tissue damage and long-term effects. By FACS surface staining, we show that the death-inducing receptors Fas, Siglec-9 and CD89 are less expressed on immature neutrophils compared to mature, activated neutrophils. To confirm this finding in functional experiments, we sorted isolated neutrophils by discriminating CD10- and CD10+ neutrophils. Their viability was assessed by AnnexinV/PI staining and the production of reactive oxygen species (ROS) was measured in both subsets.

P69 ROLE OF VINCULIN IN NEUTROPHIL ADHESION AND TRAFFICKING.

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Neutrophils must traffic from the circulation to extravascular tissue sites to resolve bacterial and fungal infections. Understanding how neutrophils migrate is critical for developing strategies to modulate inflammation to the benefit of the host. Neutrophils do not typically form mature focal adhesions, but do express the focal adhesion protein vinculin. The role of vinculin in adhesion and motility has been characterized in mesenchymal and other slow moving cell types, where vinculin organizes and regulates adhesion dynamics by virtue of its association with actin and with many cytoskeletal adaptor proteins. However, its involvement in leukocyte trafficking remains unclear. This study characterizes the role of vinculin in neutrophil adhesion, motility, deformation and mechanics. While intrinsic activation of beta-2 integrins is unaffected by vinculin knockout, we observe that neutrophils lacking vinculin have attenuated adhesion and spreading under static conditions, but not in the presence of shear stress. We further probed vinculin-deficient neutrophil mechanics and found that vinculin plays a role in generation of traction forces, mechanosensing substrate stiffness, and modulation of neutrophil deformation. Despite these defects, vinculin-deficient neutrophils were able to traffic as well as wild-type neutrophils in a mouse model of acute peritonitis. Together, our ongoing studies point to a role for vinculin in modulating neutrophil adhesion that appears to be dependent on the mechanical context in which it occurs.

P70 SHEAR-DEPENDENT SHEDDING OF TETHERS FROM ROLLING AND ARRESTED NEUTROPHILS PRODUCES ELONGATED MICROPARTICLES: CANDIDATE BIOMARKER FOR SEPSIS

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Human plasma contains 0-1400/μl elongated particles (median length 5.4(1.7-23)μm, width <0.5μm) that are positive for human neutrophil (hPMN) surface markers (CD16 and CD66b) but negative for the microparticle markers CD9, CD63, CD81 and Annexin5 as detected with confocal microscopy and Image Stream (Amnis). 24 septic and 20 healthy blood samples revealed 4-fold increase of elongated particle count in plasma of septic patients (705(0-1400)/μl) compared to healthy controls (115(0-420)/μl). The elongated particle count was robust and remained unchanged when plasma was stored at 4C for up to 5 days. Flow chamber experiments showed that hPMN rolling on P-selectin or arrested on ICAM-1 and IL-8 shed tethers (elongated particles) under physiological shear stress (20-40dyn/cm2). Tethers were visualized by labeling with anti CD16-AF647 mAb. Mouse neutrophils (mPMN) isolated from bone marrow and labeled with anti Ly6G-AF647 mAb also shed tethers. Mouse cremaster intravital imaging confirmed the presence of neutrophil-derived elongated particles in the plasma upon trauma induced neutrophil rolling and arrest, where we observed tether detachment from rolling mPMN and tether shedding from arrested mPMN.

P71 *Bothrops moojeni* VENOM AND BMOO-LAAO MODULATED THE NEUTROPHILS ACTIVATION BY REDUCING CXCL8/IL-8; CCL2/MCP-1 PRODUCTION AND INCREASING CD11B EXPRESSION

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Bothrops snake venoms are complex mixtures of biologically active components, such as toxins, peptides, and enzymes, including L-amino acid oxidases. These components induce a significant accumulation of leukocytes at sites of tissue injury, characterized by early neutrophil infiltration and inflammatory process. We tested the direct immunomodulatory potential of Bothrops moojeni crude venom (BmV) and its L-amino acid oxidase (Bmoo-LAAO) on the expression of surface activation markers (CD66b and CD11b) and chemokine production (CXCL8/IL-8; CCL2/MCP-1; CCL5/RANTES; CXCL9/MIG; CXCL-10/IP-10) in human neutrophils. The neutrophils were cultured in the presence of BmV or Bmoo-LAAO during 16-18h. Then, cell viability by PI staining, expression of cell activation markers and chemokine production were determined. BmV at concentrations of 50 and 75 ug/mL reduced CXCL8/IL-8 and CCL2/MCP-1 production (p<0.05). The Bmoo-LAAO (at 50 and 75 ug/mL) was capable of reducing only the CCL2/MCP-1 (p<0.05). These effects were accompanied by the CD11b upregulation (Bmoo-LAAO 50 and 75 p<0.01; BmV 50 p< 0.05 and 75 p<0.01) and CD66b downregulation (BmV 50 and 75 p<0.0001). BmV and Bmoo-LAAO did not have any effect on CCL5/RANTES, CXCL9/MIG and CXCL-10/IP-10 production by neutrophils. Since the CD11b, CXCL8/IL-8 and CCL2/MCP-1 levels are associated with autoimmune and inflammatory diseases pathogenesis these results open new perspectives for exploring the potential of BmV and Bmoo-LAAO as immunotherapeutic agents for inflammatory diseases treatment. Supported by FAPESP 2011/23236-4

P72 HOW DOES INTERFERON GAMMA (IFNγ) IMPROVE INNATE IMMUNE FUNCTION IN HUMAN LEUKOCYTES?

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This study aims to identify if IFNγ can restore neutrophil function including phagocytosis and bacterial killing and to elucidate the signalling pathways involved. Neutrophils freshly isolated from healthy volunteers with in vitro induced dysfunction, using salbutamol, were treated with IFNγ and their phagocytic and bacterial killing ability were investigated. Results showed a 20% decrease in phagocytosis by neutrophils when exposed to salbutamol (P <0.001), with phagocytic function restored when incubated with IFNγ (P =0.003) by reinstating RhoA activity. Bacterial killing of Pseudomonas aeruginosa and Staphylococcus aureus was inhibited with salbutamol, with a 50% reduction in killing. IFNγ significantly restored neutrophil ability to kill all bacterial strains used, except methicillin-resistant Staphylococcus aureus (MRSA). Using specific inhibitors, we show that the restoration of neutrophil function by IFNγ uses Janus kinases 1&2 (Jak1&2) and signal transducer and activator of transcription 1 (STAT1) pathways and is phosphatidylinositol-3-kinase (PI3K)-dependant, involving both the γ- and δ-isotypes. Western blotting experiments showed that 3-phosphoinositide-dependent kinase 1 (PDK1) activity was reduced while phosphatase and tensin homolog (PTEN) activity restored, following IFNγ treatment. Providing that we can validate these results in neutrophils isolated from ICU patients, who tend to present neutrophil dysfunction, IFNγ could potentially become a viable therapeutic option.

P73 AN INTEGRIN-DRIVEN BASIS FOR THE NEUTROPHILIC RESPONSE TO CANDIDA

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Systemic fungal infections such as those caused by Candida sp. are particularly problematic in patients maintained in the surgical ICU for extended periods of time. As with other tissue-based anti-microbial responses, neutrophils emigrate from the circulation and navigate within the extracellular matrix of the afflicted tissue until direct contact with the infectious agent is achieved. Since Candida hyphae are too large to engulf, other host defense mechanisms such as NET release take on the added significance for combating physically large extracellular pathogens. Moreover, the neutrophil response to tissue infection must occur in the presence of matrix, therefore we questioned whether the anti-fungal response of neutrophils is regulated by integrins. We found that the β2 integrin, CR3 (Complement Receptor 3, αMβ2), functions as a pattern recognition receptor for the fungal cell wall component β-glucan, thereby expanding the functional repertoire of integrin engagement in immune defense. We show the regulatory role of two members of the β1 integrin family, VLA3 (α3β1) and VLA5 (α5β1), in determining the matrix-dependent response to Candida. We present data in support of the hypothesis that the β2 integrin CR3 regulates the activation status of both VLA3 and VLA5 by an intracellular path of integrin cross-talk. CR3 leads to up-regulation (or activation) of VLA3 and the co-incident down-regulation (or suppression) of VLA5. VLA3 and VLA5 regulate distinct neutrophil functions with VLA3 determining swarming/clustering and VLA5 determining NETosis. We establish the significance of β2-to-β1 integrin cross-talk in controlling anti-candida effector functions of neutrophils, and determine the necessity of the extracellular matrix protein, fibronectin, for clustering and NETosis.

P74 NEUTROPHIL NADPH OXIDASE DEFICIENCY LIMITS INFLAMMATION INDUCED BY FUNGAL PAMPS.

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The NOX2 NADPH oxidase generates superoxide following activation by microbial or inflammatory stimuli. Inactivating mutations result in chronic granulomatous disease, associated with recurrent bacterial and fungal infections as well as dysregulated inflammation. This includes the response to fungal cell wall pathogen-associated molecular patterns (PAMPs), which is typically neutrophilic. Whether NADPH oxidase-derived reactive oxygen species (ROS) generated by neutrophils play a crucial role in regulating inflammation, in addition to their microbicidal effect, is still elusive. The objective of this study was to examine whether deficient neutrophil ROS are directly linked with hyperinflammation induced by fungal PAMPs. In vitro and in vivo studies were conducted using mouse models. We confirmed that NADPH oxidase-null mouse neutrophils produce substantially higher levels of CXCL2, TNF-α and IL-1β compared to WT neutrophils upon in vitro challenge with the sterile yeast particle, zymosan. To interrogate the relative role of the neutrophil NADPH oxidase in the response to fungal PAMP-induced inflammation in vivo, we developed mice with lineage–restricted reduction in neutrophil NADPH oxidase activity using the S100A8-Cre recombinase. Neutrophils exhibited 10-20% of wild-type NADPH oxidase activity whereas monocyte and macrophage activity was intact. Instillation of zymosan into lungs induced acute neutrophilic inflammation that was substantially greater in NADPH oxidase-null mice, as reported. Inflammation in mice with S100A8-Cre-mediated depletion of neutrophil NADPH oxidase, although less compared to NADPH oxidase-null mice, was significantly greater than wild-type mice. Thus, the neutrophil NAPDH oxidase plays a crucial role in limiting the inflammatory response to fungal PAMPs.

P75 CHARACTERIZATION OF THE SIGNALING PATHWAY INVOLVED IN THE NEGATIVE REGULATION OF NEUTROPHIL ACTIVATION BY THE MYELOID INHIBITORY RECEPTOR CLEC12A.

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Clec12A is an inhibitory receptor that is involved in the pathogenesis of gout. Gout is one of the most painful types of arthritis caused by recurring inflammatory episodes in which neutrophils (PMNs) play a key role. When PMNs come into contact with monosodium urate crystals (MSU), the expression of Clec12A diminishes. Decrease in Clec12A causes an enhanced PMNs response. Although Clec12A contributes to the immunopathogenesis of gout, we still do not know how Clec12A signals. The aim of the project is to characterize the Clec12A signaling pathway. We stably transfected 293T cells with HA-tagged, wild-type Clec12A and a Clec12A mutated in its ITIM motif. We developed an antibody against the phosphorylated form Clec12A's ITIM motif to track its phosphorylation status, (R-94P). Standard cell biology approaches were used to study the subcellular localisation of Clec12A and its signaling. The 293 cells stably expressing Clec12A can signal through this receptor. Phosphorylated Clec12A was detected with R-94P in 293T cells transfected. Moreover, SHP-2 is recruited to the phosphorylated ITIM of Clec12A. Receptor engagement induces the translocation of Clec12A to detergent-resistant membrane domains (DRMs) where it becomes phosphorylated and internalised. We developed an in vitro system and new antibody to study how Clec12A signals. Our findings reveal that Clec12A signals within DRMs. We also identified proteins involved in the Clec12A signaling pathway. Together, our observations provide insight into the pathways involved in gout and the molecular mechanism through which Clec12A dampers inflammation.

P76 THE PERIODONTAL PATHOGHEN, FILIFACTOR ALOCIS, FAVORS RELEASE OF ANTI-INFLAMMATORY NEUTROPHIL-DERIVED CYTOKINES OVER PRO-INFLAMMATORY WHEN COMPARED TO OTHER ORAL BACTERIA.

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Filifactor alocis has emerged as an important periodontal pathogen. The effect of F. alocis challenge on human neutrophil cytokine and chemokine gene and protein expression was tested. Total RNA was isolated from highly purified neutrophils after 1-3-6 h of F. alocis challenge. Six chemokines and 3 cytokines which were differentially regulated by RNAseq were validated by qPCR. Bacteria-free supernatants were collected after 24 h from unstimulated cells or stimulated with Pam3CSK4,FSL1, F. alocis, or other oral bacteria (Streptococcus gordonii, Porphyromonas gingivalis, Peptoanaerobacter stomatis);chemokine and cytokine levels were determined by Luminex or ELISA. RNAseq and qPCR showed peak levels of CXCL1,CXCL2,CXCL3, CXCL8, CCL3, CCL4,TNFα,IL1-β and IL-1ra mRNAs expression in neutrophils after 1 h of F. alocis challenge. The mRNA expression levels of CXCL1 and CCL4 were higher compared to the other chemokines. P. stomatis induced the highest release of CXCL1. P. gingivalis induced higher release of CXCL8 compared to F. alocis and S. gordonii. Except for P. gingivalis, all other oral bacteria induced robust release of IL-1ra. Bacteria-free conditioned supernatant (cond-sup) was tested for neutrophil and monocyte chemotaxis. Only P. stomatis cond-sup induced neutrophil and monocyte chemotaxis.Cond-sup from P. gingivalis and S. gordonii induced neutrophil and monocyte chemotaxis, respectively. In conclusion, except for IL-1ra, neutrophils produce lower levels of chemokines and cytokines in response to F. alocis but not to other oral bacteria.

P77 INVESTIGATING THE FUNCTIONAL AND PHYSICOMECHANICAL PROPERTIES OF THE DEPRIMED NEUTROPHIL

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Healthy pulmonary vasculature plays an important immunomodulatory role, where it appears to retain primed neutrophils (Nϕ), releasing them into the circulation in a quiescent, or deprimed, state. Failure of this mechanism in lung injury is thought to play an important role in extra-pulmonary organ dysfunction. Here we investigate the functional and mechanical phenotype of a deprimed Nϕ.

Methodology: Nϕ were primed for 5 mins with PAF or vehicle control for all experiments, and deprimed for 2 hrs. L-selectin and CD11b surface expression were evaluated using flow cytometry. ROS-generation was evaluated using chemiluminescence. Systemic (HMVEC-D) and pulmonary microvascular endothelial cells (HMVEC-P) were grown in Ibidi® 0.4μm channels for flow interaction. Mechanical properties were evaluated using real-time deformability cytometry (RTDC).

Results: PAF priming results in rapid L-selectin loss and CD11b up-regulation. L-selectin remains low in deprimed Nϕ (p0.003), whilst CD11b returns to control levels (p0.127). PAF priming and fMLP stimulation results in a significant increase in ROS production (p< 0.0001). Depriming results in similar ROS production to controls (p0.99). There is a significant increase in adherence to HMVEC-D (p<0.0001) and HMVEC-P (p0.0001) in PAF-primed Nϕ. There is a selective decrease in adherence of deprimed Nϕ to HMVEC-P (p0.99) but not to HMVEC-D (p0.99, p0.5).

Conclusions: Deprimed Nϕ are functionally and rheologically similar to unprimed ones, with some notable differences. They express lower levels of L-selectin and, importantly, they are released easily by the pulmonary endothelium, but appear to adhere to the systemic endothelium.

P78 INFLUENCES OF EARLY LIFE EVENTS ON FUNCTIONAL MATURATION OF PHAGOCYTES

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There is increasing interest in the role of the microbiota on extra-intestinal immunity, including systemic contexts of autoimmunity and pathogen defense. We previously demonstrated the role of gut-derived bacterial peptidoglycan in priming function and governing half-life of adult circulating phagocytes. Significantly less, however, is understood about microbiota-induced systemic immunity during postnatal life, when the microbiota is first acquired. Considering microbial communities during early life (EL) remain in a state of constant flux and are highly dynamic, we propose that fluctuations in microbial community structures during EL modulates functionality of EL phagocytes, which in turn affects pathogen susceptibility. To determine effect of age on innate immunity, we characterized bone marrow (BM) and splenic phagocytes in infant and adult mice via flow cytometry. At steady state, surface expression of CD11b, a cellular activation marker, is significantly increased on EL phagocytes. We also found that EL neutrophils exhibited surface marker phenotypes reminiscent of circulation-aged neutrophils in adults, which have higher phagocytic and inflammatory activity. To gauge phagocyte functionality, we assessed uptake capacity by EL and adult BM mouse phagocytes ex-vivo. Bacterial uptake was significantly increased in EL phagocytes, corroborating our finding that phagocyte activation is enhanced during EL. Preliminary experiments using antibiotic treatments support a role of the microbiota in modulating EL phagocyte activation status. Administration of vancomycin, gentamicin and ampicillin through mothers resulted in lower CD11b expression, but not Ly6C or Ly6G, in EL BM phagocytes. Our results suggest EL phagocytes, contrary to expectation, are in a more poised, activated state.

P79 LOW DENSITY GRANULOCYTES DEMONSTRATE NON-REGULATORY ROLE IN SYSTEMIC LUPUS ERYTHEMATOSUS

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The presence of pro-inflammatory low density granulocytes (LDG) has been demonstrated in autoimmune and infectious diseases. Recently, regulatory neutrophilic myeloid derived suppressor cells (PMN-MDSC) were identified in systemic lupus erythematosus (SLE). Since, LDG and PMN-MDSC share similar phenotype with contrasting functional effects, we explored these cells in a cohort of subjects with SLE.

Results: LDG prevalence was elevated in SLE vs healthy controls, associated with the type 1 interferon gene signature and disease activity. SLE LDGs exhibit significantly heightened expression of multiple activation markers including CD63. Supernatants from SLE LDGs did not restrict healthy CD4+ T-cell proliferation in an arginase-dependent manner, suggesting LDGs are not immunosuppressive. Indeed, the CD4+ T-cells produced significantly higher levels of pro-inflammatory cytokines (IFN-g, TNF-a and LT-a) than those exposed to SLE NDG supernatants.

Conclusions: Based on our results, while LDGs express high levels activation markers, they exert pro-inflammatory effects on T-cells and do not exhibit MDSC function.

P80 TISSUE SPECIFIC NEUTROPHIL ACTIVATION STATES IN HEALTH AND INFLAMMATION.

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Neutrophils are quickly recruited to tissues in response to pro-inflammatory cues, however little is known about tissue neutrophil phenotypes in health. The objective of our study is to identify unique tissue neutrophil activation states through immunophenotyping of neutrophils from various healthy and inflamed tissues in the mouse. We used a multicolour flow cytometric approach to assess cluster of differentiation (CD) marker expression on neutrophils from mouse bone marrow, blood, peritoneum, spleen, liver, fat, colon and oral cavity. Neutrophils were also assessed after acute peritonitis, DSS-induced colitis and ligature-induced periodontal disease. Fixation prior to sample processing and labeling was performed in order to preserve cell surface CD marker signatures in their native states. Our results demonstrate that a low level of “sentinel” neutrophils occur in various sterile and non-sterile tissues of naïve mice. We found that tissue resident neutrophils from peritoneum, fat, oral cavity and colon each have unique CD marker expression signatures in health, and inflammation-induced neutrophil immunophenotypes were also unique to each tissue and source of inflammation. We conclude that considerable variability of tissue neutrophil activation states exists in health and during inflammation.

P81 ANHYDROGLUCITOL-CORE GALLOTANINS FROM RED MAPLE (ACER RUBRUM) MODULATE METABOLISM AND OXIDATIVE BURST OF ACTIVATED HUMAN BLOOD NEUTROPHILS

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Anhydroglucitol-core gallotannins (ACGs) are phenolic compounds only found in maple species. We reported that 100 µM of ginnalin A (GinA) and ginnalin 3,6 (Gin3,6), ACGs from red maple (Acer rubrum), induce apoptosis of human blood neutrophils. The objective of this study was to establish the effects of GinA and Gin3,6 on metabolism and oxidative burst of neutrophils activated with phorbol 12-myristate 13-acetate (PMA) or the cytokines TNF and IL-1beta. Metabolic parameters (glycolysis and oxygen consumption) were assessed with the Agilent/ Seahorse Bioscience XFe96 Extracellular Flux Analyzer, the oxidative burst was determined by flow cytometry using the CellROX® dye. GinA and Gin3,6 reduce PMA- and cytokine induced-glycolysis up to 60% and 70%, respectively. The oxygen consumption rate was lowered by more than 70% when cells were stimulated with TNF and IL-1beta, but not with PMA. GinA and Gin3,6 decreased the oxidative burst by more than 60% for PMA and cytokine stimulations. In parallel, GinA and Gin3,6 decreased viable cells stimulated by TNF and IL-1beta by 20% while increasing early apoptotic neutrophils by 10%. In conclusion, ACGs such as GinA and Gin3,6 can significantly affect glycolysis, oxygen consumption and oxidative burst of human blood neutrophils in vitro, suggesting that such ACGs could be useful to modulate the involvement of neutrophils in chronic inflammatory diseases.

P82 ANHYDROGLUCITOL-CORE GALLOTANNINS FROM RED MAPLE (ACER RUBRUM) INDUCE APOPTOSIS OF HUMAN BLOOD NEUTROPHILS

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Apoptosis of neutrophils is an essential checkpoint for the resolution of inflammation by shutting down the deleterious functions of these immune cells. This study investigated the role of anhydroglucitol-core gallotannins (ACGs) in the increase of apoptosis previously reported for human blood neutrophils treated by the hot water extract from red maple buds (RMB). Fractions obtained by liquid-liquid partitioning of this extract were assessed for their effects on neutrophil viability by flow cytometry. Major compounds of these fractions were purified and checked for their effects on neutrophil apoptosis. Mixtures of pure compounds were also assayed to evaluate interaction effects. The apoptosis-related proteins in neutrophil lysates were analyzed by using human apoptosis antibody array kit. Ethyl acetate and butanol fractions that contained the major ACGs ginnalin A, ginnalin 3,6 and ginnalin C were shown to stimulate neutrophil apoptosis. These ACGs at 100 μM significantly increased the rate of late apoptotic cells. When differentially combined, these ACGs have additive or antagonist effects related to their concentrations in the mixtures studied, especially for ginnalin C. Pro-apoptotic effects of the ACGs were associated with significant increases of FADD, phospho-Rad17 and SMAC/Diablo. Deepen experiments on these compounds could be useful for the assessment of their potential in the development of novel therapeutic approaches that facilitate resolution of neutrophil-mediated inflammatory diseases.

P83 COMPARISON OF TWO METHODS FOR NEUTROPHIL CONCENTRATES PRODUCTION

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Objective. At the request of French Blood Center (EFS) we analyzed 3 neutrophil functions on preparations issued from two different methods of neutrophil collection and concentration (apheresis, currently method and super-mix) to consider if both were comparable. EFS needs to switch to supe-mix method briefly.

Methods. Experiments (n=30) were analyzed by flow cytometry: viability by using 7-AAD dye, reactive oxygen species (ROS) production by hydro ethidium oxidation and adhesion molecules expression with anti CD11b, CD18 and CD62L antibodies. ROS production and adhesion molecules were measured before and after stimulation with TNF, LPS and fMLF.

Results. Viability was higher in neutrophils from apheresis than those from super-mix (99% vs 97% p<0.05). Expression and modulation of adhesion molecules after stimulation were higher in super-mix, particularly after stimulation by TNF (p<0.05 for CD11B and CD18). ROS production was higher in super-mix with fMLF with or without pre-stimulation by TNF or LPS (p<0.05).

Conclusion. Comparison of the two methods shows a higher neutrophil viability from apheresis method, but viability of super-mix neutrophils is still good. Neutrophils from super-mix seems basally pre-activated because they produce high amounts of ROS without TNF or LPS priming and the expression of adhesion molecule is higher than those from apheresis without any stimulation. We concluded that neutrophils from super-mix shows a high activated phenotype that could be useful on transfusing critical neutropenic patients.

P84 NEUTROPHILS RAPIDLY PRODUCE TH2 CYTOKINES IN RESPONSE TO LARVAL BUT NOT ADULT HELMINTH ANTIGEN

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Initiation of immune responses to helminth infection require IL4 receptor signaling, activating STAT6 and resulting in elevated interleukin (IL) -4 and -13 production. This phenomenon occurs during early Haemonchus contortus (Hc) infection in parasite-resistant St. Croix (STC) sheep, yet the cell responsible for early cytokine production is unknown. Studies report greater infiltration of neutrophils to the abomasum of STC by 3 days after Hc infection as compared to parasite-susceptible Suffolk sheep (SUF). Neutrophils have been reported to play a role in immune responses to murine helminth infections and were found to produce IL-13, which promoted alternatively activated macrophage differentiation thus, it is reasonable to hypothesize that neutrophils are an early source of IL-4/IL-13 that promote resistance to Hc infection. This study aimed to determine ovine neutrophil cytokine production when stimulated with antigen (Ag) from larval and adult stages of Hc. Neutrophils from STC and SUF sheep were co-cultured with larval (HcLA) or adult (HcWA) Ag. Supernatant IL-4/IL-13 were measured using an ovine-specific enzyme-linked immunosorbent assay (ELISA). Neutrophils from either breed exposed to HcLA produced significantly higher levels of IL-4 by 30 minutes (STC, 3153.65 pg/ml and SUF, 4665.22 pg/ml) and IL-13 by 6 hours (STC, 391.02 pg/ml and SUF, 419.6 pg/ml) when compared to neutrophils cultured with HcWA (STC IL-4, 6.04 pg/ml and SUF, 8.05 pg/ml, respectively) (STC IL-13, 10 pg/ml and 12.5 pg/ml) (P < 0.001). While no breed differences were observed, these data indicate that neutrophils preferentially respond to HcLA compared to HcWA, implicating neutrophils as a potential effector cell responsible for initiation of early Th2 responses during H. contortus infection in sheep.

P85 SPLEEN TYROSINE KINASE IS CRITICAL FOR NEUTROPHIL RESPONSES TO PATHOGENIC FUNGI

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Invasive fungal infections constitute a lethal threat to patients, with mortality as high as 90%. This increasing incidence stems from an expanding use of immunomodulatory agents -chemotherapy, biologics and transplantation - that increase our patient's susceptibility to infectious complications.

Neutrophils are the initial innate cellular responders to many types of pathogens, including invasive fungi. As a testament of their critical role in controlling fungal pathogens, approximately ten percent of individuals with neutropenia become infected with yeasts such as Candida or molds such as Aspergillus. A central process for innate immune recognition of fungi is through lectin binding receptors, many of which rely on spleen tyrosine kinase (syk) for cellular activation. Neutrophils express several of these lectin receptors, including Dectin-1. We previously demonstrated that syk activation is essential for cellular activation, phagosomal maturation and elimination of phagocytosed fungal pathogens.

Here we use syk-specific small molecule inhibitors to demonstrate that neutrophils rely on syk for elimination of several Candida species. In addition, we verify these observations through the use of a conditionally-immortalized ER-Hoxb8 Cas9-expressing neutrophil cell lines to generate syk-deficient neutrophils. Syk-deficient neutrophils are unable to control C. albicans or C. glabrata but, in contrast, kill C. auris. Interestingly, neutrophil responses to Candida include the production of reactive oxygen species and cytokines such as TNF-alpha, and these are dependent on syk. On the other hand, phagocytosis and neutrophil swarming are functions that differ in response to certain Candida species through a syk-dependent process. These results suggest that syk can tune neutrophil fungicidal activity to precise fungi.

P86 RESISTANCE OF MYCOBACTERIUM SMEGMATIS TO OXYGEN-DEPENDENT KILLING BY NEUTROPHILS

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The bacterium Mycobacterium tuberculosis is a major human pathogen. Despite well-established diagnostic tests and inexpensive treatment, the incidence of tuberculosis remains high and multidrug resistance is becoming a serious problem. In order to develop new treatment methods, we need to better understand how M. tuberculosis evades the immune system. Recent evidence implicates the neutrophil as a potential site for bacterial replication. Our aim was to study neutrophil killing of the related but non-pathogenic mycobacterium M. smegmatis. Reagent hypochlorous acid (HOCl) killed M. smegmatis but a seven times higher dose was required to kill this bacterium compared to Staphylococcus aureus. Neutrophils phagocytosed M. smegmatis but killing was slow and not blocked by an NADPH oxidase inhibitor. Using a fluorescent probe for HOCl we demonstrated rapid production of this oxidant by neutrophils phagocytosing M. smegmatis, but a myeloperoxidase inhibitor did not interfere with bacterial killing. Our results suggest that M. smegmatis protect themselves from HOCl generated within the neutrophil phagosome. How they do so is the subject of ongoing work.

P87 CLEAVED N-TERMINAL HISTONE TAILS DISTINGUISH BETWEEN NADPH OXIDASE (NOX)-DEPENDENT AND NOX-INDEPENDENT PATHWAYS OF NEUTROPHIL EXTRACELLULAR TRAP FORMATION

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Neutrophil Extracellular Trap (NET) formation was originally described as a NADPH oxidase (NOX)-dependent pathway. However, it appears there are also NOX-independent pathways of NET release. We aimed to develop a serological method allowing the discrimination between NETs generated through NOX-dependent or NOX-independent pathways.

Histones from in vitro generated NOX-dependent and NOX-independent NETs were characterized with a panel of lupus-derived antibodies against N-terminal histone tails. NETs in patients with various autoimmune diseases were characterized in sandwich ELISAs employing antibodies against myeloperoxidase (MPO) and N-terminal histone tails.

We found that neutrophil elastase cleaves the N-terminal tails of core histones during NOX-dependent, but not during NOX-independent NET formation. Consequently, the detection of MPO - histone complexes with antibodies against N-terminal histone tails allows discrimination between NETs formed through a NOX-dependent or NOX-independent manner. Characterization of in vivo circulating NETs revealed the presence of NOX-independent NETs in Rheumatoid Arthritis, Systemic Lupus Erythematosus and sepsis, but NOX-dependent NETs in Psoriatic Arthritis. These results indicate heterogeneity in NET-forming pathways in vivo and highlight the need for disease-specific strategies to prevent NET-mediated pathology.

P88 BIOFILM À LA CARTE: THE QUORUM-SENSING MOLECULE AHL-12 IS RECOGNIZED VIA THE BITTER RECEPTOR T2R38 ON MYELOID CELLS

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Bacterial biofilms are nowadays considered the most common cause of persistent infection. In order to coordinate biofilm formation, bacteria use quorum-sensing molecules, also known as autoinducers. Previous studies showed that phagocytic cells might also recognize these molecules and might therefore inhibit biofilm formation at an early stage. Our focus in this study was on the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone (AHL-12), because it is also known as an 'interkingdom signalling molecule', which means that it also interacts with mammalian cells. Tissue samples of patients suffering from bone infections were evaluated. Myeloid cells were isolated from blood and from bone marrow. Investigations were performed using immunohistochemistry, cytofluorometry, western blot and laser scan microscopy. We found that AHL-12 activates phagocytes via a rather specialized receptor that was not previously described on myeloid cells, the bitter taste receptor T2R38. Taste receptors are commonly associated with cells of the gustatory system. The extragustatory expression, however, suggests an additional role, namely the sensing of the onset of bacterial biofilm infection.

P89 ACTIVATION OF NEUTROPHILS BY THE EXTRACELLULAR POLYMERIC SUBSTANCE OF S. EPIDERMIDIS BIOFILMS IN IMPLANT-ASSOCIATED INFECTIONS

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Implant-associated infections are still one of the most feared complications in the field of orthopaedics. Bacteria attach to the implant surface and form socalled biofilm colonies. Previously, we were able to demonstrate that the immune system, and neutrophils in particular, recognize biofilms which leads to an infiltration of phagocytic cells, as well as to a production of pro-inflammatory and osteolysis-inducing cytokines. In this study we addressed the question, how neutrophils recognize bacterial biofilms. Staph. epidermidis biofilms were grown and the biofilm extracellular substance (EPS) was extracted. Activation of neutrophils was evaluated by up-regulation of CD11b and CD66b, generation of oxygen radicals and release of DNA. We found that the protein fraction of the EPS activated neutrophils. Subsequently, we identified the bacterial heat-shock protein GroEL as a likely candidate. GroEL is present in the EPS and depletion of GroEL from the EPS reduced neutrophil activation. Culture of neutrophils with recombinant GroEL up-regulated CD11b and CD66b surface expression and induced oxygen radical production. Furthermore, EPS and GroEL induced DNA-release in neutrophils. The TLR4 pathway appeared to be crucial for the EPS-induced up-regulation of CD11b and CD66b, but not for induction of oxygen-radical production; suggesting involvement of additional receptors. In conclusion, we identified the bacterial heat-shock protein GroEL as an activator of the local innate immune response in biofilm infections.

P90 ROLE OF INTRACELLULAR S100A8/A9 IN THE REGULATION OF NEUTROPHIL PRO-INFLAMMATORY FUNCTIONS USING THE MYELOID HOXB8 CELL LINE

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S100A8 and S100A9 are two Ca2+-binding proteins abundantly expressed in the cytosol of neutrophils. These proteins are tightly associated to the pro-inflammatory functions of neutrophils and are now described as damage-associated molecular pattern. When released, S100A8/A9 contribute to the amplification of the inflammation process by recruitment and activation of neutrophils and other inflammatory cell types. At the intracellular level, although it is well established that S100A8/A9 regulate the NADPH oxidase activity, there is no evidence for their implication in other key functions of neutrophils. Thus in the following work, we are committed to study the role of intracellular S100A8/A9 in the degranulation process. For that purpose, we used conditional immortalized murine myeloid progenitors derived from WT and S100A9-/- mice. First, we characterized the neutrophilic phenotype and functions of WT and S100A9-/- cells. As expected, both cell types acquire a neutrophil phenotype after 5 days of differentiation and a maximal fMLF-induced ROS production is observed in WT, while reduced in S100A9-/-. After LPS-stimulation, the expression of CD markers specific for gelatinase granules, secretory vesicles and specific granules were up-regulated at the plasma membrane of WT cells, whereas decreased in S100A9-/-. These preliminary results using the Hoxb8 immortalized murine myeloid progenitors suggest that S100A9 is involved in the LPS-induced degranulation process. In this context, potential new roles of S100A8/A9 in the neutrophil inflammatory response and their associated mechanisms (e.g. protein activation, cytokine secretion) could provide new insight of their involvement in the pathogenesis of inflammatory diseases.

P91 DETECTION OF HYPOCHLOROUS ACID IN NEUTROPHIL PHAGOSOMES WITH THE PROBE R19S

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It is well accepted that neutrophils use oxygen-dependent mechanisms to kill microbes. Specific details of oxidative killing remain the subject of debate. There is strong evidence that the combined action of the NADPH-oxidase and myeloperoxidase converts oxygen to hypochlorous acid (HOCl), which is toxic to all microbes. Studying HOCl production within phagosomes is challenged by the inaccessibility of the subcellular compartment and the short lifetime of this reactive oxidant. To overcome these challenges, we have used R19S, a fluorescent probe that is proven to be sensitive for imaging HOCl. The objective of this study was to use R19S to further our understanding of oxidation reactions inside neutrophil phagosomes. We prove that when HOCl reacts with R19S, there is formation of a chlorinated product R19S-Cl in addition to the fluorescent species R19. In experiments with neutrophils, we used flow cytometry and mass spectrometry to measure the production of R19 and R19S-Cl after phagocytosis of zymosan or S. aureus. We show that during phagocytosis, R19S is oxidized to R19 as well as R19S-Cl by a process dependent on myeloperoxidase and chloride. Real-time production of HOCl was tracked by live cell imaging after phagocytosis. Wide variability was observed in the lag-time of individual phagosome formation and the onset of fluorescence. This suggests heterogeneity in the initiation of HOCl production in phagosomes. After fluorescence started to appear, however, there was tight uniformity in the increase of the signal, denoting manifest HOCl production. We conclude that oxidation of R19S provides further evidence that HOCl is formed inside phagosomes. Our work supports a purposeful role of HOCl production in neutrophils as a toxic agent against ingested microbes.

P92 THE C5A-LTB4 AXIS IN BULLOUS PEMPHIGOID DISEASES – OPENING THE DOOR FOR GRANULOCYTES INTO THE SKIN

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Massive recruitment of granulocytes into the skin is a hallmark of pemphigoid diseases. The molecular mechanisms overcoming the endogenous protection systems of skin homeostasis to initiate and broadly amplify granulocytes recruitment into the skin are still largely elusive. We have recently found evidence that the recruitment of granulocytes into the skin is initiated and perpetuated by the action of the complement fragment C5a – leukotriene B4 (LTB4) axis. Thus, in models of pemphigoid diseases, neutrophil recruitment into the skin was completely disrupted and mice were, consequently, dramatically resistant to disease, when only one of the mediators of the C5a-LTB4 axis or their receptors were lacking, highlighting the C5a-LTB4 axis as most promising therapeutic target for the treatment of pemphigoid diseases. New drug compounds inhibiting one or the other of the two mediators or simultaneously inhibiting both are about to be licensed, thus, opening new avenues for the treatment of pemphigoid diseases in the near future. These upcoming principles will be discussed.

P93 PLATELET-NEUTROPHIL CROSSTALK: SEROTONIN MEDIATED NEUTROPHIL ACTIVATION

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Studies in mice and humans have shown that neutrophil recruitment to sites of acute inflammation is modulated by plasma serotonin (5-HT). While 5-HT is believed to promote a pro-inflammatory phenotype, the mechanisms of 5-HT mediated neutrophil activation remain unknown. Our aim was to identify 5-HT activation pathways in neutrophils.
5-HT plasma levels and neutrophil activation was analyzed in samples from patients with acute myocardial infarction (MI) and from patients treated with 5-HT re-uptake inhibitors (SRIs) and from healthy donors. Neutrophils were isolated and screened for 5-HT receptors (5-HTR) by qPCR. Specific 5-HTR antagonism was performed on whole blood samples. Plasma 5-HT and MPO levels in MI patients strongly correlated with the expression of integrin alphaM (CD11b) on neutrophils 24 h post MI (Pearson r=0,846 and r=0,6606, respectively). Long term SRI treatment reduced plasma 5-HT by 50%. Neutrophil CD11b and plasma MPO levels were reduced in these patients. Screening for 5-HTR on neutrophils revealed the expression of 5-HTR, most prominently receptor subtypes 4 and 7. Whole blood stimulation with 5-HT induced increased CD11b expression on neutrophils within minutes, whereas other integrins were not affected. This was associated with the release of MPO. Treatment of blood with a 5-HTR7 antagonist inhibited any stimulatory effect mediated by 5-HT. We identified a novel pathway of neutrophil activation via 5-HT and consecutive degranulation pathways. Our findings suggest a pro-inflammatory effect of 5-HT on neutrophils during acute inflammation. Antagonism of 5-HT mediated neutrophil activation might offer new strategies for treatment of inflammatory diseases s. a. ischemia reperfusion.

P94 THE COMBINATION OF AZITHROMYCIN AND FLUTICASONE DECREASES AIRWAY NEUTROPHILIA IN AN EQUINE MODEL OF ASTHMA

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Severe asthmatic patients with airway neutrophilic inflammation are poorly responsive to conventional therapy with inhaled corticosteroids (IC). Macrolides are used as an add-on medication for these patients, owing to their anti-inflammatory properties targeting neutrophils. Macrolides have been shown to improve quality of life and the exacerbation rate in asthmatics, but their effects on lung function and lower airway neutrophilic inflammation are controversial. Heaves is a naturally occurring disease of horses with marked similarities to human asthma including lower airway obstruction, mucus hypersecretion, pulmonary remodeling and airway neutrophilia. We aimed to determine if addition of azithromycin would potentiate the therapeutic effects of IC in severe asthma. Severe asthmatic horses were administered inhaled fluticasone (2500 ug twice daily) alone (n=6) or combined with oral azithromycin (10 mg/kg, q48h; n=6) for 5 months. Lung function, tracheal mucus accumulation and bronchoalveolar lavage fluid cytology were sequentially measured (at baseline, after two weeks of therapy (T2), T4, T6, T8, T12, T16, T20). Data were analyzed with two-way ANOVA and Dunnett's for multiple comparisons. Pulmonary resistances and elastances similarly improved in both groups of horses with therapy. There were group and time effects on airway inflammation with the combination of azithromycin and fluticasone significantly reducing neutrophilia from T4 to T20 while fluticasone alone decreased it only temporarily at T8 and T12. The tracheal mucus accumulation was unchanged during the trial. This study shows that the addition of a macrolide to IC can reduce neutrophilic inflammation in severe asthma, without potentiating the improvement of lung function.

P95 THE ER-HOXB8 SYSTEM: A MODEL OF CONDITIONAL MYELOID DIFFERENTIATION FOR THE IN VITRO AND IN VIVO STUDY OF NEUTROPHIL AND MONOCYTE DEVELOPMENT AND FUNCTION

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Neutrophils and monocytes are critical first responders of the innate immune system. Their study is hindered by the lack of good model systems and the difficulties in isolating primary cells. Here we update the ER-Hoxb8 system of conditional myeloid differentiation that has been adopted by more than 75 labs world-wide. ER-Hoxb8 is a fusion protein that joins the estrogen receptor (ER) hormone binding domain to the Hoxb8 transcription factor. The protein is constitutively expressed though it is active only in the presence of estradiol (like the normal ER). Introducing ER-Hoxb8 into bone marrow mononuclear cells leads to continuous self-renewal and permits the unlimited expansion of murine granulocyte-monocyte progenitors (GMPs). These GMPs can then be synchronously and terminally differentiated upon withdrawal of estradiol. Progenitor lines are factor-dependent. Lines can be derived in media supplemented with one of Flt3-ligand, SCF, IL-3, or GM-CSF. The vast majority of our work (and others) has been done in clones derived in SCF. Here we show data to demonstrate the key advantages of this system in the study of neutrophils and monocytes:
1. The progenitors are diploid and non-transformed
2. One can produce unlimited numbers of cells on demand
3. Clonal lines can be derived for 100% purity with no isolation steps
4. The progenitors are genetically-tractable (protein overexpression, shRNA, CRISPR/Cas9 manipulation)
5. The mature cells are terminally-differentiated
6. The mature cells are functional in vitro: cytokine & superoxide production, phagocytosis, pathogen-killing
7. The cells are functional in vivo: they can be transfused into recipient mice where they home to the sites of infection and protect against live pathogen-challenge.

P96 DISRUPTION OF THE NEUTROPHIL PI3 KINASE AND PTEN SIGNALING AXIS BY TREPONEMA DENTICOLA M.M.

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Neutrophils in the oral cavity are in close contact with a number of bacterial species including Treponema denticola. T. denticola modulates neutrophil activity, but how the signaling pathways are altered is poorly understood. The outer membrane sheath protein (Msp) of T. denticola inhibits neutrophil chemotaxis through disruption of the phosphoinositide 3-kinase (PI3K)/ Phosphatase and tensin homolog (PTEN) axis; which appropriate activation is crucial in generation of phosphoinositide signaling molecules. PTEN inhibits the PI3K pathway, which is inappropriately activated by Msp. PTEN can be regulated by many mechanisms including phosphorylation. Our objective is to determine how T. denticola and Msp interfere with the different PI3K p110 catalytic subunits (alpha, beta, gamma and delta) and PTEN regulatory mechanisms. The ability of 3 Msp isotypes to differentially manipulate the PI3K/PTEN axis was also investigated. We hypothesized that treatment of neutrophils with bacteria or Msp may decrease the phosphorylation state of PTEN, thus activating the molecule and contributing to the inhibition of neutrophil chemotaxis. Murine bone marrow neutrophils were treated with T. denticola strains or purified Msp and expression of PI3K subunits and PTEN phosphorylation were assessed by western blot. Changes in PTEN and PI3K subunit gene expression was assessed using real-time PCR. We found that treatment with both Msp and bacteria decreased the amount of the PI3K delta subunit, which is normally highly expressed in neutrophils and crucial in chemotaxis. Likewise, PTEN phosphorylation was decreased; which may reflect a conformational change leading to PTEN activation. We conclude that T. denticola Msp modulates PI3K and PTEN regulation, thus further defining the mechanism of how T. denticola dampens neutrophil response.

P97 ARF6 INVOLVEMENT IN NEUTROPHIL FUNCTIONS.

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Objective:Neutrophil (PMN) recruitment to inflammatory sites is a tightly regulated process. At these sites PMNs perform their functions, including reactive oxygen species (ROS) production. Then PMNs undergo apoptosis, to ensure the resolution of inflammation. PMNs sense their environment through various receptors that propagate the signals inside the cells via small GTPase regulatory proteins such as Arf6. In this study we made use of PMN-specific Arf6 knockout (KO) mice to evaluate the impact of Arf6 depletion on PMN functions in vivo and ex vivo.
Methods: Inflammation was induced by injection of 500 ng LPS or fMLF (10-7 M) into the air pouches. PMNs collected 4h after injection of LPS were used in ex vivo studies. Surface expression of b2-integins and phagocytosis of non-opsonised/opsonised fluorescent zymosan A were monitored using flow cytometry. Cytochrome c and Luminol assays were used to measure superoxide (O2-) and ROS production. Air pouch PMN apoptosis was measured after culture in the presence or the absence of 10% foetal bovine serum (FBS) for 24h using the Annexin V/PI viability assay.
Results:LPS and fMLF-mediated recruitment of PMNs into air pouches was reduced in the Arf6 KO mice. Reduced PMN migration correlated with reduced cell surface expression of b2-integins. Phagocytosis of zymosanA (opsonised and non-opsonised), as well as fMLF-mediated production of O2- and ROS were decreased in PMNs of Arf6 KO mice. Furthermore, deletion of Arf6 differently impacted PMN apoptosis, depending on the presence or the absence of FBS.
Conclusion: Arf6 plays a significant role in PMN migration to sites of inflammation in vivo through modulation of cell surface expression of b2-integins. Arf6 signalling regulates several PMN functional responses (phagocytosis, ROS and O2- production) and their fate toward apoptosis.

P98 ENHANCED TUMOR IMMUNE SURVEILLANCE THROUGH NEUTROPHIL RE-PROGRAMMING DUE TO TOLLIP DEFICIENCY

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Although tumor immune environment is increasingly recognized to play a key role in the modulation of tumorigenesis and tumor regression, most of the attention related to tumor-immune therapies in the past has been paid to adaptive immune cells. The role and regulation of innate leukocytes such as neutrophils during the modulation of tumor immune environment remain controversial and less clearly defined. Here we observed that the selective deletion of Toll-interacting protein (Tollip), a key innate immune cell modulator, renders enhanced tumor immune surveillance in a chemically induced colon tumor animal model. Tollip deficient neutrophils significantly elevate T cell proliferation trough enhanced expression of co-stimulatory molecule CD80, and reduced expression of inhibitory molecule PD-L1. Mechanistically, Tollip deficiency increases STAT5 levels and reduces STAT1, responsible for the expression of CD80 and PD-L1 respectively in neutrophils. Through adoptive transfer study, we demonstrate that Tollip deficient neutrophils are sufficient to transfer enhanced tumor immune surveillance and reduce tumor burden in vivo. Our data reveal a novel re-programming strategy of neutrophil function conducive for enhancing anti-tumor immune environment through reducing Tollip.

P99 IMMUNE COMPLEX-INDUCED NEUTROPHIL APOPTOSIS IS DISTINCT FROM PHAGOCYTOSIS INDUCED CELL DEATH

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Background: Immune complexes (ICs) potently activate neutrophils to induce functions that include phagocytosis, reactive oxygen species generation, production of cytokines, and release of inflammatory mediators. Circulating neutrophils are recruited to sites of infection. Phagocytosis of pathogens promotes neutrophil apoptosis in a process called phagocytosis-induced cell death (PICD). We recently showed that insoluble immune complexes (iICs) also cause neutrophil apoptosis using a non-canonical signalling pathway, FcgR-PI3Kb/d-Cdc42-Pak-Mek-Erk. Here we investigate the mechanism of iIC-induced neutrophil apoptosis.
Methods: Neutrophils were isolated from peripheral blood of healthy volunteers. They were stimulated with iICs or with particles such IgG-opsonised zymosan or latex beads, in the presence or absence of different inhibitors. Induction of apoptosis was assessed by flow cytometry and immunofluorescence was used to investigate internalisation of ingested particles.
Results: We show here that neutrophil apoptosis is induced with IgG-opsonised particles or iICs. As part of this, neutrophils engulf iICs and also, zymosan and IgG-opsonised latex beads. However, the underlying mechanism is not the same. (i) The signalling pathways employed are not the same. (ii) Blocking iIC internalisation does not abolish iIC induced apoptosis. (iii) Inhibiting PI3K prevents internalisation of iICs but not that of (small) IgG-opsonised particles.
Conclusion: iIC-induced neutrophil apoptosis and PICD are separate events that are regulated by separate pathways. It will be interesting to test in the future whether they serve different physiological pathways.

P100 DYSREGULATION OF G-CSF BY NEUTROPHILS MAY CONTRIBUTE TO CHRONIC INFLAMMATION IN AGING RHESUS MACAQUES

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Neutrophils are the major cells produced via bone marrow hematopoiesis and need to be continuously replenished from the hematopoietic stem cells throughout life. How aging impacts the kinetics and regulation of neutrophil production in vivo is still not clear. Using in vivo 5-bromo-2'-deoxyuridine (BrdU) pulse-chase labeling, we recently demonstrated the consistent kinetics of neutrophils in healthy rhesus macaques (RMs) between 3 – 19 years of age. We also found a significant negative correlation between daily neutrophil production and age, but the half-life of these cells remained unchanged (He et al., J. Immunol. in press). In the present study, intriguingly, we observed a significant decrease in bone marrow transit time and higher in-group variability of neutrophil kinetics in older RMs between 20-24 years old. We also measured plasma cytokines, including granulocyte colony-stimulating factor (G-CSF), in relation to neutrophil numbers in a cross-sectional study in healthy RMs (n=137) between 2 - 24 years old. We observed that chronological aging negatively correlated with the neutrophil numbers in blood but positively correlated with plasma G-CSF, suggesting a dysregulated compensatory mechanism for neutrophil production in the aging animals through higher production of G-CSF. Plasma G-CSF levels also positively correlated with concentrations of plasma IFN-g, TNF-a, IL-1b, IL-1ra, IL-4, IL-12. Overall, our results demonstrated a shift and higher variability in neutrophil kinetics in animals above 20 years old. The results also raise questions about whether the chronic increased G-CSF levels are an attempt to restore neutrophil kinetics homeostasis in elderly RMs.

P101 DIFFERENTIATED HL-60-DERIVED MICROVESICLES AS A DRUG DELIVERY VEHICLE

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HL-60 cells, a promyeloid leukemia cell, can be differentiated into neutrophil-like cells which are key effectors in the host's immune response. At the site of infection, neutrophils release large amounts of microvesicles (MVs) which contains various molecules such as cytokines, proteases, and miRNAs. However, it has not been fully determined whether neutrophil-like differentiated HL-60 cell release MVs. In the current study, we investigated the formation of MVs from neutrophil-like differentiated HL-60 cell (dHL-60) and further identified the possible use of dHL-60-derived MVs as a drug delivery vehicle. HL-60 cells were differentiated to neutrophil-like cells with the treatment of all-trans retinoic acid (ATRA). ATRA-treated HL-60 cells showed neutrophil-like morphology and surface marker expression. Moreover, the dHL-60 cells showed increased NET and ROS generation in response to PMA stimulation compared to naïve HL-60. dHL-60 cells produced more amounts of MVs than naïve HL-60 cells when stimulated with fMLP. Interestingly, dHL-60-derived MVs did not show significant effects on the bactericidal activity against bacteria nor the chemotaxis of monocytes, suggesting dHL-60-derived MVs as an attractive vehicle for drug delivery. Therefore, we transfected dHL-60 cells with penicillin, antibiotics against gram-positive bacteria. Penicillin-loaded dHL-60-derived MVs showed significant bactericidal activity against S. aureus. These results suggest that dHL-60-could be loaded with therapeutic targets to derive cargo loaded MVs, and thus can be an attractive target for drug-delivery system.

P102 THE IMPORTANCE OF SERINE PROTEASES FOR NEUTROPHIL FUNCTION AND INFLAMMATORY TISSUE DAMAGE IN THE ORAL CAVITY

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Neutrophil serine proteases (NSP), elastase, proteinase 3 and cathepsin G are the most potent proteases produced in neutrophils. They are believed to be central for neutrophil antimicrobial activities by contributing to phagosomal killing and degradation, but also to be culprits of inflammatory tissue damage. To become enzymatically active, the NSPs require proteolytic processing by the exo-cysteine protease cathepsin C (CTSC). In humans, loss-of-function of CTSC results in a rare disease known as Papillon-Lefèvre Syndrome (PLS) with neutrophils devoid of NSP activity. PLS patients suffer from extensive tissue damage with a particularly aggressive type of periodontitis, but they are surprisingly not susceptible to opportunistic bacterial infections. The main questions are why NSPs are not essential to combat infections and why their absence induces destructive inflammation. By utilizing a small cohort of PLS patients, we have started to explore the roles of NSP activity on neutrophil functions and the regulation of inflammation. We have confirmed that the absence of CTSC activity leads to a lack of NSP activity in neutrophils. In comparison to healthy neutrophils from blood, PLS neutrophils were fully normal regarding oxygen radical production, degranulation and cell death regulation in vitro, but had reduced capacity to form extracellular traps in response to certain common triggers. Oral samples (gingival exudates) from PLS patients contained similar levels of neutrophils as samples from general chronic periodontitis, which argues against NSPs being essential for neutrophil chemotaxis. Currently, we are exploring the impact of NSPs on the degradation of inflammatory and homeostatic mediators in the gingival crevice.

P103 EXPERIMENTAL HUMAN ENDOTOXEMIA RESULTS IN ACCELARATED MATURATION OF THE NEUTROPHIL COMPARTMENT IN THE HUMAN BONE MARROW.

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Neutrophils are thought to be short-lived cells and are produced in extremely high numbers in the bone marrow (>1011 cells/day). Nevertheless, solid data to support this hypothesis is lacking. We investigated the proliferation and maturation of the neutrophil compartment in humans during homeostasis and experimental human endotoxemia, as in vivo model for acute systemic inflammation. Bone marrow and blood were obtained from healthy volunteers before and during acute systemic inflammation evoked by intravenous challenge with endotoxin (lipopolysaccharide, 2 ng/kg) . By differential CD16/CD11b staining promyelocytes, myelocytes, metamyelocytes, banded cells and mature cells were identified and sorted for analysis on cytospins. Differentiation was also studied with a phenotyping panel, containing Ab against CD305, CD49d, , CD16, CD62L, CD11b, CD35, CD66b, CD13, CD11c and CD10. Four hours after endotoxin-challenge, the neutrophil compartment exhibited signs of accelerated maturation, characterized by an increased number of cells with more mature characteristics in all neutrophil fractions. Although large numbers of banded neutrophils were found in the peripheral blood, these cells in the bone marrow did not decrease. Also, the number of CD62Llow neutrophils increased in both blood and bone marrow. In conclusion, our study shows that the neutrophil compartment in the bone marrow of healthy volunteers responds immediately to systemic inflammatory cues and shows signs of accelerated maturation. During short term inflammation, the release of neutrophils with banded or hypersegmented nuclei does not lead to depletion of these cells in the bone marrow.

P104 USE OF COMMON ANTI-CD16 ANTIBODIES FOR FLOW CYTOMETRY HAVE A FUNCTIONAL EFFECT ON HUMAN NEUTROPHILS

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Introduction: CD16 (FcγRIII) is a receptor expressed on neutrophils and monocytes. Antibodies against CD16 are routinely used in flow cytometry of neutrophils. We aimed to validate anti-CD16 for use in live cell flow cytometry to ensure that they did not alter the cellular phenotype of freshly isolated neutrophils. This was achieved by measuring the surface expression of multiple targets, including markers of activation and cell viability using annexin V and 7AAD.

Methods: Neutrophils from healthy young donors were isolated from whole blood using discontinuous Percoll gradients, before incubation for 20 minutes with primary antibodies, followed by 15 minute incubation with annexin V. 7AAD dye was then added to cells were before flow cytometry analysis on a BD Fortessa X20.

Results: Incubation of freshly isolated, live neutrophils with a common clone (3G8) of anti-CD16 antibody appeared to induce apoptosis, as measured by increased binding of annexin V. Furthermore, surface expression of PD-L1 was also increased, detected by changes in the median fluorescence intensity of anti-human PD-L1. Further analysis using different manufacturers and clones of anti-CD16 demonstrated a reproduceable induction of PD-L1 expression on the surface of neutrophils.

Conclusion: These data highlight that common clones of anti-CD16 antibodies have functional effects on neutrophils, after only a short period of time. In our study, the use of anti-CD16 impacted the observed phenotype of neutrophils and therefore may impact cellular function. This is an important consideration for studies where anti-CD16 antibodies are being used in functional assays, or live cell flow cytometry.

P105 CIRCULATING PLATELET-NEUTROPHIL AGGREGATES REPRESENT A PERIPHERAL BIOMARKER OF TYPE 1 DIABETES (T1D) DEVELOPMENT IN NOD MICE.

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In certain autoimmune diseases, platelets and neutrophils are engaged in a vicious cycle of activation which promotes autoimmune disease. The aim of this study was to examine the contribution of platelet-neutrophil interactions to T1D development in NOD mice. Platelet-neutrophil aggregates (PNAs) in the peripheral blood of NOD female mice at 3, 4, 6-8, 10-12, 16-18 weeks of age and at T1D onset and in insulitis leukocytes (harvested from islets isolated from mice at the corresponding ages) were analysed by flow cytometry. PNAs were identified as CD41+ve (platelet marker) Ly6Clo/Ly6Ghi neutrophils. Peripheral blood of male NOD mice at 3 -18 weeks of age (with characteristic low T1D incidence) and non-autoimmune control B6.SL mice were analysed in parallel. Three waves of circulating PNAs were found in female NOD mice, peaking at 4 weeks, 10-12 weeks of age and at T1D-onset. These peak PNA levels correlated with the initiation of T1D autoimmunity in young NOD mice (3 weeks of age), marked infiltration of islets by inflammatory leukocytes (at 10-12 weeks) and the clinical onset of T1D. In contrast, male NOD mice demonstrated an elevation in PNAs only at 4 weeks of age. Islet PNAs were found to peak in NOD females at 4 weeks and 10-12 weeks of age. Islet PNAs therefore characterised the initiation of T1D autoimmunity in NOD mice (3 weeks) and contributed to the intra-islet leukocyte influx to damage beta cells in female mice at 10-12 wks. In female NOD mice the dynamic profile of islet-associated PNAs correlated with the PNA profile in peripheral blood. These findings suggest that PNA levels in peripheral blood represent a novel biomarker of T1D development.

P106 NEUTROPHIL-DERIVED MICROVESICLES AND TRAILS ARE FUNCTIONALLY DIFFERENT EXTRACELLULAR VESICLES.

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Activated neutrophils release different types of extracellular vesicles with diverse biological functions. Neutrophils during migration or diapedesis into inflammatory foci deposit chemokine-containing extracellular vesicles known as trail which guide the migration of following virus-specific CD8+ T cells. When neutrophils arrived at the inflammatory foci, they release microvesicles that induces aggregation of bacteria leading to arrest of bacterial growth. Although the diverse functions ranging from the immune modulation to antimicrobial activity have been studied, the specific characterization of these neutrophil-derived extracellular vesicles has not been fully understood. Thus, we evaluated the differences between two major neutrophil-derived extracellular vesicles, microvesicles and trails. The stimulation of neutrophils with chemoattractants, cytokines, and bacteria induced the production of microvesicles and trails from neutrophils. Microvesicles (identified as Annexin V+ CD16hi vesicles) and trails (identified as Annexin V+ CD16lo vesicles) showed the similar composition of contents and surface markers. Further, both mircovesicles and trails showed direct bactericidal activity and induced chemotaxis of monocytes. However, microvesicles and trails showed different effects on the phenotype polarization of macrophages and survival of septic mice. Neutrophil-derived extracellular vesicles were also detected in the serum of healthy donors and their number was significantly increased in the serum from septic patients. Together, our study suggests the important insights into the understanding the neutrophil-derived extracellular vesicles.

P107 IL-17a PRODUCTION IN CLOSED EYE TEAR NEUTROPHILS IN HEALTH AND DISEASE

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Introduction: Interleukin-17a (IL-17a) signaling is important in pathogenesis of ocular surface disorders, specifically in dry eye disease. Production of IL-17a has been mainly attributed to T cells, though prior art has shown that IL-17a may be produced by neutrophils in certain disease states. This study sought to understand IL-17a production by closed eye neutrophils both in normal subjects and in dry eye disease.

Methods: Eight normal and eight dry eye human participants were recruited and trained to wash the ocular surface with phosphate buffered saline for at-home self-collection of tear leukocytes immediately following a full night of sleep (closed eye). Tear leukocytes were isolated and counted and peripheral blood was collected. Unstimulated blood and tear leukocytes were incubated with a panel of fluorescently-labeled antibodies to distinguish T cells and neutrophils, and for the exclusion of dead cells, B cells, monocytes, and natural killer cells. Intracellular production of IL-17a was assessed using the transcription factor buffer set (BD Biosciences; San Jose, CA). Flow cytometry was performed using a BD LSR II.

Results: IL-17a was measurable within both normal and dry eye closed eye tear neutrophils. On average, normal subjects (9.1 ± 9.9%) had about twice as many IL-17+ neutrophils as dry eye subjects (4.4 ± 3.5%), though this did not achieve statistical significance (p = 0.27). This was significantly higher (p < 0.01) than the amount of IL-17a detected in blood-isolated neutrophils from normal (0.23 ± 0.17%) and dry eye subjects (0.26 ± 0.23%).

Conclusions: IL-17a is produced by closed eye tear neutrophils both in homeostatic conditions and in dry eye disease.

P108 ORAI1 AND ORAI2 CALCIUM CHANNELS REGULATE NEUTROPHIL CALCIUM SIGNALING AND HOST DEFENSE

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Calcium is a signaling molecule that transduces signals downstream of a diverse array of receptors. The primary mechanism by which calcium signals are initialed in immune cells is by the process of store-operated calcium entry (SOCE). Receptor activation triggers transient calcium release from the endoplasmic reticulum, which is followed by opening of plasma membrane calcium-release activated calcium (CRAC) channels. To determine the role of CRAC channel proteins Orai1 and Orai2 in neutrophil activation we generated mice with hematopoietic deletion of Orai1 and/or Orai2. In vitro, the loss of Orai1 results in decreased calcium influx compared to wild type neutrophils. Furthermore, loss of both Orai1 and Orai2 nearly abolishes store-operated calcium influx. Decreased SOCE in Orai1- and Orai1/2-deficient neutrophils impairs key bactericidal functions including phagocytosis and ROS production. Studies by others have demonstrated that Orai2 is a negative regulator of SOCE in other immune cells. In contrast, we find that Orai2-deficient neutrophils display decreased calcium influx and impaired ROS production. To determine the role of Orai-mediated calcium entry in vivo, we used a skin infection model with methicillin-resistant Staphylococcus aureus (MRSA), a pathogen that is highly susceptible to killing by neutrophil ROS. Mice lacking both Orai1 and Orai2 develop significantly larger lesions with delayed healing compared to WT mice. These results demonstrate a unique role for Orai2 in neutrophil SOCE which contrasts with its known role in other immune cells and identify a critical role for Orai1 and Orai2 in neutrophil calcium signaling, bactericidal functions and host defense.

P109 EVALUATION OF HOW HUMAN PHAGOCYTES RESPOND TO LIGANDS DIRECTED TOWARDS THE FREE FATTY ACID RECEPTORS GPR84 AND FFA2R

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Objective: The objective was to characterize basic phagocytic functions by the recently de-orphanized G protein-coupled receptor (GPCR) GPR84, recognizing medium chain fatty acids. GPR84 has been suggested to play an important role in inflammatory reactions.

Methods: By utilizing human phagocytes (neutrophils, monocytes and monocyte-derived macrophages; MDMs), we have measured how GPR84 ligands (agonist – ZQ16 and a GPR84 selective antagonist) can induce intracellular Ca2+ transients and upregulation of granule-localized receptors by flow cytometry, reactive oxygen species (ROS) by chemiluminescence and, chemotaxis using a Boyden filter assay system. Further, the response to the GPR84 specific ligands by in vivo transmigrated skin-chamber neutrophils have been examined. All responses have been related to ligands directed towards FFA2R, a GPCR that recognizes short chain fatty acids.

Results: Compared to FFA2R, which is functionally expressed by neutrophils but not monocytes, GPR84 is expressed by both cell types and also by MDMs. Regarding neutrophils, the activation profile of GPR84 and FFA2R was very similar, with low ROS release by naïve cells, which could be significantly primed by TNF and by the actin cytoskeleton disrupting agent Latrunculin A. Our data also suggests that both GPR84 and FFA2R use a desensitization mechanism that bypasses the actin cytoskeleton and that both GPR84 and FFA2R are involved in the neutrophil recruitment process in vivo.

Conclusion: Our study reveals functional similarities but also some significant variances between GPR84 and FFA2R in human phagocytes, and thereby provide new mechanistic insights into how GPR84 is regulated by phagocytes.

P110 TRAPPING NEUTROPHILS INSIDE MAST CELLS AT SITES OF ANAPHYLAXIS

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Neutrophils are key immune sentinel cells, which are recruited from the bloodstream to local sites of tissue inflammation and infection. Intravital microscopy (IVM) studies in mice over the last decade have helped us to understand how neutrophils navigate in the interstitial tissue and exert their effector functions outside the vasculature, mainly in models of sterile inflammation and infection. However, very little is known about the dynamic behavior of neutrophils in anaphylactic tissues. By combining IVM with a model of passive cutaneous anaphylaxis, we made the surprising observation that neutrophils are attracted to degranulating mast cells, and interacted with them for several hours. Detailed analysis of this process by state-of-the-art live cell imaging in vitro revealed that neutrophils become completely trapped inside living mast cells, forming a novel cell-in-cell structure, which we term “Mast cell Intracellular Trap” (MIT, MC-Trap). Our work defines the detailed sequence of events and molecules leading to the formation of this novel form of emperipolesis. Inside MITs, neutrophils escape apoptosis and survive for several days, before they eventually die inside mast cells. Functional assays and transcriptome analysis point to a beneficial role of MIT formation for mast cell recovery after degranulation, with potential implications for the course of anaphylactic reactions and chronic inflammatory conditions.

P111 NEUTROPHIL SERINE PROTEINASE ACTIVITY IN ALPHA-1 ANTITRYPSIN DEFICIENCY

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Neutrophil elastase (NE) has long been considered the main proteinase responsible for lung damage in Alpha-1-antitrypsin deficiency (AATD). However, recent evidence has suggested that Proteinase 3 (PR3) may play an even more important role in the pathophysiology of emphysema. PR3 is present at three times the concentration of NE in the neutrophil azurophilic granules and will only be inhibited by AAT after NE. We have developed Europium-based sandwich ELISAs based on specific fibrinogen cleavage products for both NE (Aalpha-Val360) and PR3 (Aalpha-Val541) activity in vivo. The purpose of this study was to determine the levels of both markers in plasma from stable AATD patients. We tested 181 individuals with the ZZ genotype plus 94 who were SZ and compared levels to 52 age matched healthy controls.

Concentrations of the NE peptide were increased (p<0.0005) in both the ZZ and SZ patients (median=14.5nM; IQR=11.8-18.3nM and 14.1nM; IQR=11.1-20.3nM respectively) compared to controls (median=9.2nM; IQR=7.3-11.5nM). However, the PR3 peptide was present in greater concentrations in all deficient subject groups (p< 0.0005 between each group) being highest in ZZ patients (median=409.3nM; IQR=240.4-644.3nM) compared to SZ patients (77.61nM; IQR=52.0-114.9nM) and lowest in control subjects (36.3nM; IQR=19.8-52.7nM). The footprint of PR3 activity is increased in ZZ patients compared to SZ AATD but not the footprint of NE activity. AAT levels in these two patient groups may provide the same (though reduced) protective barrier against NE mediated tissue damage. However, in ZZ individuals the lower AAT levels provide less protection against PR3 associated damage which may account for the reduced susceptibility of SZ subjects to cigarette induced neutrophilic damage.

P112 THE EFFECT OF YOPH ON SIGNAL TRANSDUCTION PATHWAYS AND ANTIMICROBIAL RESPONSES IN NEUTROPHILS DURING YERSINIA PSEUDOTUBERCULOSIS INFECTION

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PMNs are the first line of host defense against many bacterial pathogens yet many of the signaling pathways that regulate their antimicrobial responses are incompletely understood. Yersinia pseudotuberculosis (Yptb), successfully evades neutrophil killing through the production and translocation of effector proteins, called Yops, which can directly interfere with signal transduction pathways in neutrophils and their biological functions. YopH is a tyrosine phosphatase that plays a key role in Yptb survival mouse infection. It blocks activation of SKAP-2 and SLP-76; these proteins play critical roles in ROS production by PMNs. Our objective is to determine whether the inhibition of ROS production by YopH is critical for Yersinia survival in infected tissues, and if so, how YopH inhibits ROS production. Here we report that YopH is essential to enhance Yptb survival in tissues of infected ROS-defective and SKAP-2-/- mice. Using neutrophils isolated from Yptb-infected lungs, we demonstrated ex vivo that Yop-translocated PMNs are unable to produce ROS in comparison to their non-translocated neighbors. Using purified ligands to stimulate bone marrow-derived PMNs in vitro, we show that YopH can completely block ITAM-associated receptors, integrin-, and Fc-mediated ROS production, and also alters fMLP-activated ROS generation. These findings indicate that SKAP-2 is involved in mediating full responses of neutrophils to a number of ITAM-mediated signal transduction pathways leading to ROS and that YopH inactivates these responses completely to prevent neutrophil ROS production enhancing the pathogenesis of Yptb.

P113 TAMOXIFEN INDUCES DEPOLARIZATION OF THE MITOCHONDRIAL MEMBRANE AND CASPASE ACTIVATION IN EQUINE NEUTROPHILS

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Neutrophils participate in innate immunity as the first line of host defense against microorganisms and allergens. However, exacerbated neutrophil activity can be harmful to surrounding tissues: this is a problem in diverse diseases including allergic asthma and chronic obstructive pulmonary disease in humans, and equine asthma (previously known as “heaves”), a disease characterized by airway remodeling and neutrophilic inflammation. Previous studies in horses with acute lung inflammation indicate that treatment with tamoxifen, a selective estrogen receptor modulator, produces a significant decrease in bronchoalveolar lavage fluid (BALF) neutrophil content and a concomitant improvement in clinical status and pulmonary function. Results showed that tamoxifen induces early apoptosis in vitro and in vivo in granulocytic cells from peripheral blood and BALF. Moreover, our data shows that tamoxifen has an inhibitory action on respiratory burst, chemotaxis and phagocytosis in equine neutrophils in a dose-dependent manner. However, the molecular mechanisms of the beneficial effect of tamoxifen on affected horses´ airway inflammation remain unclear. In this study, through techniques of immunodetection and flow cytometry, we show that tamoxifen depolarizes the mitochondrial membrane and activates caspase 3 in equine neutrophils in vitro. Results show that tamoxifen induces neutrophil caspase 3 activation within twenty minutes of co-incubation, and also induced depolarization of the mitochondrial membrane from thirty minutes of co-incubation onwards. In summary, these results contribute to our knowledge about the mechanisms involved in activation of neutrophil apoptosis and suggest that tamoxifen is able to active the intrinsic apoptotic pathway, improving the clinical symptomatology of horses affected with airway.

P114 15-LIPOXYGENATION OF FATTY ACIDS BY HUMAN NEUTROPHILS.

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Neutrophils and eosinophils are important sources of bioactive lipids from the 5- and the 15-lipoxygenase (LO) pathways. While eosinophils are recognized to express large amounts of 15-LO-1, the expression of 15-LO-1 and 15-LO-2 in neutrophils was not irrefutably established, notably because most studies involving neutrophils were performed in absence of eosinophil depletion. Herein, we isolated neutrophils and eosinophils from the peripheral blood of healthy and rhinitic volunteers, respectively. Cells were then treated with a cocktail of fatty acids. Oxilipins from the 15-LO pathway were then quantitated by LC-MS/MS.
Neutrophils and eosinophils metabolized linoleic acid into 13-HODE, dihomo-gamma-linolenic acid into 15HETrE, arachidonic acid into 15-HETE, eicosapentaenoic acid into 15-HEPE, docosahexaenoic acid into 14- and 17-HDHA and arachidonyl-ethanolamide into 15-HETE-EA. In neutrophils, the synthesis of 15LO metabolites was rapid and reached a plateau (1 to 15 min). This was not the consequence of enzyme inactivation but rather linked to a balance between 15-LO metabolite synthesis and degradation. Although neutrophils and eosinophils both synthesized 15-LO metabolites, we observed differences in substrate preference. We also found that neutrophils expressed the 15-LO-2, in contrast with eosinophils which expressed the 15-LO-1. Finally, while selective 15-LO-1 inhibitors blocked 15-LO activity in eosinophils, they did not in neutrophils.
Our data show that human neutrophils have the ability to synthesize several 15-LO metabolites, independently of 15-LO-1 and very likely via the 15-LO-2. This latter pathway might contribute to the regulation of inflammation, by controlling the levels of neutrophil-derived 15-LO metabolites.

P115 MODULATING NEUTROPHIL RESPONSES WITH MANUKA HONEY

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Objective: Ascertain the effect of Manuka honey on neutrophil response using an in vitro model cultured under various inflammatory and anti-inflammatory environments.
Methods: The effect of Manuka honey on neutrophil response was analyzed using a differentiated human promyelocytic cell line (dHL-60) model. dHL-60s were exposed to a variety of inflammatory (fMLP, LPS, IFN-gamma, GM-CSF, and combinations) and anti-inflammatory (TGF-beta, IL-4, IL-13, and combinations) stimuli in the presence of 0, 0.5, and 3% Manuka honey (Medical grade 12+) for 3 and 24 hours. Culture supernatants were assayed using a MAGPIX immunoassay for the inflammatory signals TNF-alpha, IL-8, IL-1beta, MIP-3alpha, MIP-1alpha, MIP-1beta, RANTES, and MCP-1, the matrix metalloproteinases MMP-9 and MMP-1, the growth factors FGF-13, VEGF, and HGF, and the anti-inflammatory factors IL-1ra, IL-12, and IL-4, as well as PRTN-3.
Results: The results show a trend of 0.5% honey reducing the release of TNF-alpha, MMP-9, MMP-1, FGF-13, IL-1beta, IL-12, MIP-1alpha, MIP-1beta, MCP-1, and RANTES, while increasing PTRN-3, HGF, IL-4, IL-1ra, and IL-8 release compared to zero honey control. In contrast, 3% honey reduces release of all assayed secreted factors except TNF-α and IL-8 compared to control. These results provide a guide to the neutrophil response under different inflammatory and anti-inflammatory stimuli and indicate the nuanced ways that various concentrations of Manuka honey alter this response.
Conclusion: While the role of neutrophils has been classically considered phagocytic, recent literature shows an ability to regulate healing through released factors. This study provides evidence that Manuka honey could play a role in modulating neutrophil response in different wound environments.

P116 KICKING BUTT AND TAKING NAMES: ANTIGEN-PRESENTING NEUTROPHILS KILL PATHOGENIC FUNGI BETTER THAN CANONICAL NEUTROPHILS

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During inflammation a small proportion of neutrophils undergo differentiation into cells with features of both neutrophils and dendritic cells (DCs) and thus are called neutrophil-DC hybrids (PMN-DCs). PMN-DCs arise in pre-clinical murine models of aspergillosis, blastomycosis and systemic candidiasis. We tracked fungal killing in vivo during fungal pneumonia finding that PMN-DCs associate with and kill fungi better than do canonical neutrophils. PMN-DCs retain neutrophil functions important for killing fungi including reactive oxygen, nitric oxide, phagocytosis and extracellular traps. Additionally, because PMN-DCs can present antigen, we demonstrated that PMN-DCs present fungal antigen and promote type 1 and type 17 responses important for immunity to fungi. Because PMN-DCs promote antifungal immunity through direct killing and induction of adaptive immunity, they are important targets for adjunctive therapies. We generated PMN-DCs from a neutrophil-progenitor cell line and showed that these PMN-DCs kill fungi well compared to canonical neutrophils, particularly for Candida albicans. Finally, we adoptively transferred in vitro derived PMN-DCs into mice with lethal systemic candidiasis and observed enhanced protection against C. albicans in vivo. With the drawbacks of antifungal drugs, immune-based therapies are being sought to treat invasive fungal infections. We believe that harnessing PMN-DCs during serious fungal infections will reduce fungal burden and increase survival in patients.

P117 PERTURBATIONS OF ZEBRAFISH PHAGOCYTE RESPONSES TO C. ALBICANS REVEAL REDUNDANT PATHS FOR YEAST SPREAD

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Candida albicans is a commensal fungus of the human mucosa that can cause systemic disease and mortality in immunocompromised patients. It is unclear how this non-motile organism spreads throughout a host, so we tested three potential host-mediated mechanisms of fungal spread: movement inside phagocytes in a "Trojan Horse" mechanism, inflammation, and endothelial barrier disruption. We utilized a zebrafish model of infection to visualize yeast spread in an entire living host, tracking fluorescently marked yeast and host cells. We tested the roles of neutrophils and macrophages on yeast dissemination via chemical and genetic blockade as well as the role of blood flow on yeast movement. Neutrophils and macrophages respond to yeast and wild type C. albicans, and time lapse imaging has revealed these immune cells transporting yeast in the bloodstream supporting a "Trojan Horse" mechanism occurs. Inflammation often precedes yeast spread, however, elimination of immune cells and subsequent reduction in inflammation does not alter dissemination dynamics. This suggests that other mechanisms are involved. Time lapse imaging of the endothelium has shown yeast passing through the blood vessels into the bloodstream. When blood flow is blocked, yeast continue to traverse the endothelium, but have limited range. We propose a two-step mechanism for dissemination whereby (1) yeast move into the blood via phagocyte assistance or direct uptake into the bloodstream and then (2) travel in the blood stream to distant tissue by blood flow or phagocyte carriage. Future work can now identify specific host and fungal molecules that mediate each mode of C. albicans spread.

P118 SECRETION OF THE PHOSPHORYLATED FORM OF S100A9 FROM NEUTROPHILS IS ESSENTIAL FOR THE PRO-INFLAMMATORY FUNCTIONS OF EXTRACELLULAR S100A9

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S100A8 and S100A9 are members of the S100 family and are abundantly expressed in the cytosol of neutrophils. Mostly found under heterodimeric form, S100A8/A9 have various intra- and extracellular functions modulating the inflammatory response of neutrophils. Previously, we showed that the intracellular activity of S100A8/A9 was regulated by the phosphorylated form of S100A9. Here, we focus on the importance of this post-translational modification on its extracellular activity and its impact on the pro-inflammatory functions of neutrophils. First, we characterized the secretion of S100A8/A9 under different stimulatory conditions and analyzed the phosphorylation state of secreted S100A9. Our results on neutrophil-like differentiated HL-60 cells and purified human neutrophils show a time-dependent secretion of S100A8/A9 with a phosphorylated-state of S100A9. Moreover, our data suggest that S100A8/A9 are secreted via the process of neutrophil extracellular trap. Next, we investigated the impact of S100A9 phosphorylation on the expression and secretion of various pro-inflammatory cytokines in dHL-60 cells by real-time PCR and cytometric bead array, respectively. Our results demonstrate that only the phosphorylated form of the S100A8/A9 complex induces pro-inflammatory cytokine expression and secretion. Finally, we were able to show that S100A8/PhosphoA9 is inducing cytokine secretion through TLR4 signaling. These results give new insight into the neutrophil pro-inflammatory response to S100A8/A9 and their possible involvement in the pathogenesis of inflammatory diseases.

P119 NEUTROPHIL SWARMING RELEASES NETS AND CONTAINS MICROBIAL GROWTH

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Neutrophil swarming seals off infection and protects healthy tissue. However, the details of the novel biological process of swarming are just emerging. Current in vivo models are qualitative, low throughput, and have limited access to mediators. Here, we leverage our microscale technologies to directly quantify the containment of microbes by neutrophil swarms.

Methods: We developed an assay to study thousands of synchronized swarming processes at once. Neutrophil swarming is triggered on large arrays of live microbe clusters, for which we control geometric features such as size and spacing. Time-lapse fluorescence imaging helps monitor the microbe-neutrophil interactions.

Results: We tested the swarming of human neutrophils against live fungi like Candida albicans and Aspergillus fumigatus and live bacteria like Staphylococcus aureus. While live microbes incubated alone grew well on the arrays, neutrophil swarms significantly delayed the growth of all microbes tested. Swarms contained fungal hyphae for up to 16 hours and disruption of swarming mediators compromised the ability to contain C. albicans. NETs were formed during swarming and disruption of NETs and ROS production compromised swarming control of fungi.

Conclusions: Neutrophil swarming occurs robustly against live microbe clusters and restricts their growth. These results establish swarming as a mechanism of fungal control that warrants further investigation. Our technology provides exquisite control over conditions during swarming against live microbes. These new tools provide direct access for quantification of cellular dynamics and molecular mediators during neutrophil-microbe interactions and overcome the limitations of current in vivo models of neutrophil swarming.

P120 CHARACTERIZATION OF THE GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF) RESPONSE INDUCED DURING STREPTOCOCCUS SUIS INFECTION

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Streptococcus suis serotype 2 is an important porcine bacterial pathogen and emerging zoonotic agent of which exacerbated inflammation is a hallmark of the infection. Neutrophilic leukocytosis is an important feature of S. suis infection, a process commonly involving granulocyte-colony stimulating factor (G-CSF), a potent pro-inflammatory cytokine responsible for neutrophil proliferation and mobilization. However, the systemic production of G-CSF induced during S. suis infection, the cell types involved, and the underlying mechanisms remain unknown.

Plasma levels of G-CSF were observed to rapidly increase in a S. suis serotype 2 mouse model of systemic infection. Though dendritic cells (DCs) and macrophages (MØ) are central to S. suis-induced inflammation, little is known regarding their capacity to produce G-CSF. We demonstrated that S. suis infection of DCs and MØ results in important, yet comparable, production levels of G-CSF. Based on these results, we evaluated the role of certain S. suis virulence factors and that of the Toll-like receptor (TLR) pathway, known to be involved in S. suis recognition, in G-CSF production. Results showed that the bacterial capsular polysaccharide masks surface lipoproteins that can activate the TLR pathway in a MyD88-dependant and partially TLR2-dependant manner.

In conclusion, this study showed that S. suis induces G-CSF production in vivo and in vitro by DCs and MØ via recognition of lipoproteins by TLR2. The implication of other virulence factors and TLRs, the role of the G-CSF in vivo and its influence on neutrophils during the course of S. suis infection are currently under evaluation.

P121 FULLY AUTOMATED LOAD-AND-GO FLOWCYTOMETRY IN NEUTROPHIL ANALYSIS: THE START OF A NEW ERA.

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Changes in functional phenotypes of neutrophils can be used to predict infectious complications. Due to technical and logistical difficulties this concept is currently not applicable. A fully automated 24/7 load and go flow cytometer would provide the opportunity to design such a test. Therefore, the aim of this study was to investigate the applicability of 24/7 automated analysis of neutrophils by flowcytometry.

For proof of principle, blood was drawn from healthy controls next to the flow-cytometer. Neutrophil activation was measured by the use of the automated AQUIOS load-and-go flowcytometer. The AQUIOS is able to pierce the tube caps, add antibodies, lyse and measure the sample within 20 minutes immediately after vena puncture. Thereafter, the same blood tubes were measured every 15 minutes in the first hour and thereafter hourly. Measurements were done in presence or absence of the bacterial stimulus fMLF. Median Fluorescent intensity(MFI) was used to analyze results. A significant increase in MFI was detected in the activation markers CD35(174%(146%-213%),P=0.004), CD11b(245%(167%-524%),P=0.009) and CD11c (220%(153%-389%),P=0.008) within the first hour after vena puncture. After 3 hours an even higher increase in all activation markers was detected (CD35(222%);CD11b(378%);CD11c(316%)). Neutrophil responsiveness to fMLF was most evident at T=0 and gradually decreased over time. Neutrophil activation significantly increase in the tube shortly after vena puncture. This artificial activation coincides with a decreased responsiveness of neutrophils. For a reproducible clinical test on neutrophil functionality it is mandatory to measure neutrophil receptor markers immediately after vena puncture in a point-of-care context.

P122 NEUTROPHILS INHIBIT HIV-1 REPLICATION IN EX VIVO HUMAN TONSIL CULTURES

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Objective: Since the role of neutrophils in HIV infection is largely unexplored, we sought to study the influence of neutrophils on HIV-1 replication and CD4 T-cell depletion in a physiologically relevant ex vivo cell culture model.

Methods: HIV replication and CD4 T-cell depletion was quantified by a SYBR Green based assay to measure reverse transcriptase (RT) activity (SG-PERT) in the culture supernatant and by flow cytometry, respectively. Tonsil tissue, cultured in suspension as human lymphoid aggregate culture (HLAC) or as tonsillar tissue blocks (HLH) was infected with HIV-1 and freshly isolated neutrophils were added. NET formation was addressed by using the NETosis Assay Kit from Cayman that measures elastase activity in the supernatant.

Results: Addition of human or mouse neutrophils induced an immediate and significant reduction of HIV-1 replication and CD4 T-cell depletion in HLAC and HLH cultures. This effect depended on the amount of neutrophils added with optimal inhibition observed at a tonsil/neutrophil ratio of at least 2:1. Inhibition of HIV-1 replication by neutrophils was observed for different lab adapted strains and primary HIV-1 isolates. Supernatants of HLAC-neutrophil co-cultures did not inhibit HIV-1 replication and CD4 T-cell depletion and separating HLAC and neutrophils by a transwell abrogated the inhibitory effect of neutrophils. Interference of HIV-1 replication in HLAC by neutrophils was not associated with the formation of NETs.

Conclusion: Neutrophils can interfere with HIV-1 replication and CD4 T-cell depletion in lymphoid HIV-1 target cells in a contact dependent manner. Ongoing experiments asses the mechanism by which neutrophils hamper HIV-1 replication.

P123 DEFICIENCY OF SOCS3 LEADS TO BRAIN-TARGETED EAE BY ENHANCED NEUTROPHIL ACTIVATION VIA G-CSF/STAT3 SIGNALING

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Dysregulation of JAK/STAT signaling is associated with pathological conditions, including Multiple Sclerosis (MS). Suppressors Of Cytokine Signaling (SOCS) are a protein family of negative regulators of the JAK/STAT pathway, and SOCS3 is critical for inhibition of STAT3 activation. We previously demonstrated that deletion of SOCS3 in myeloid cells (LysMCre-SOCS3fl/fl) results in a severe, non-resolving atypical form of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. We observed preferential neutrophil infiltration in the cerebellum. However, the exact function of neutrophils in the pathology of atypical EAE is unclear. We demonstrate that SOCS3-deficient neutrophils exhibit enhanced STAT3 activation and a hyper-activated phenotype in response to G-CSF in vitro. RNA-seq and gene set enrichment analysis reveals a unique gene expression profile and several pathways related to neutrophil activation in SOCS3-deficient neutrophils in response to G-CSF. Functionally, G-CSF priming leads to increased ROS production mediated by TNF-alpha and GM-CSF and enhanced formation of neutrophil extracellular traps (NET) in SOCS3-deficient neutrophils. At the peak of EAE, neutrophils from the cerebellum of LysMCre-SOCS3fl/fl mice show an overtly activated phenotype, such as altered surface markers, excessive ROS production and degranulation. Neutralization of G-CSF in vivo significantly reduces the incidence and severity of atypical EAE, and decreases neutrophil infiltration, degranulation, and oxidative stress in the cerebellum. Overall, this work demonstrates that SOCS3 deficiency in neutrophils leads to atypical EAE by enhanced neutrophil activation via G-CSF/STAT3.

P124 NEUTROPHIL NITRIC OXIDE IS PROTECTIVE IN A ZEBRAFISH MODEL OF MYCOBACTERIAL INFECTION

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Tuberculosis is on the rise due to the increasing prevalence of multi-drug resistance. We aim to understand the host response to mycobacterial infection to identify host-derived factors as potential therapeutic targets, circumventing bacterial resistance. Macrophages have been identified as being critical regulators of TB control, but the roles of neutrophils in infection control is less clear due to a lack of suitable in vivo models. In an in vivo zebrafish Mycobacterium marinum (Mm) model, we have identified hypoxia signalling, via hypoxia inducible factor (Hif-1alpha) transcription factor, as a host-derived pathway that protects against infection. Genetic stabilisation of Hif-1alpha led to decreased bacterial burden in an inducible nitric oxide synthase (iNOS) dependent manner. Hif-1alpha stabilisation primed neutrophils with high levels of nitric oxide (NO), allowing them to better deal with infection. This effect was partially dependent on expression of the important pro-inflammatory cytokine interleukin-1beta. When macrophages are depleted, the protective effect of neutrophil NO remains, indicating that neutrophils are able to deal with mycobacterial infection, in vivo, if activated with Hif-1alpha. Our data show that Hif-1alpha and NO are important host-derived immune modulators in TB and highlight that neutrophils can help to control infection in vivo. Modulation of Hif-1alpha/NO levels may be a novel therapeutic strategy circumventing the problem of multi-drug resistance.

P125 EFFECT OF NEUTROPHILS ON OSTEOCLASTOGENESIS IN PERIODONTITIS MICE

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Periodontitis is characterized with alveolar bone resorption. We investigated the effect of neutrophils on osteoclastogenesis in neutrophil-depleted periodontitis mice.

Mice were divided into control (C), periodontitis (P), and periodontitis with anti-Ly6G Ab (P+Ly6GAb) groups. Periodontitis was induced by ligature of molar (day 0). Mice were injected by anti-Ly6G Ab before and after ligature and were sacrificed on day 3 and 7. CD11b+ and Ly6G+ neutrophils in blood were counted by FACS. TRAP+ osteoclasts and Ly6G+ neutrophils/RANKL+ neutrophils in periodontal tissue were counted after TRAP and immunohistochemical staining, respectively.

Neutrophils in blood were higher in P group than C group but were decreased in P+Ly6GAb group than C and P groups on day 3 and 7. On day 3, osteoclasts, Ly6G+ neutrophils, and RANKL+ neutrophils in periodontal tissue were higher in P group than C group. However, they were lower in P+Ly6GAb group than P group. On day 7, osteoclasts and Ly6G+ neutrophils were higher in P and P+Ly6GAb groups than C group, but there was no difference in P and P+Ly6GAb groups.

Osteoclastogenesis with infiltration of Ly6G+ neutrophils and RANKL+ neutrophils is increased in the periodontal tissue of periodontitis mice. Neutrophil depletion in blood decreases osteoclasts with RANKL+ neutrophils and Ly6G+ neutrophils in periodontal tissue on day 3, but not on day 7. These suggest the involvement of neutrophils in osteoclastogenesis through RANKL in the early stage of periodontitis. This work was supported by the National Research Foundation of Korea(NRF) grant funded by the Korea government(MSIT) (NRF-2017R1A2B4002348).

P126 REG3β REGULATES MAINTENANCE OF NEUTROPHIL GRANULOCYTES WITHIN THE ISCHEMIC HEART

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OBJECTIVE: The infiltration and activation of neutrophil granulocytes (Nphs) is a prerequisite for the initiation of endogenous healing processes after the onset of myocardial infarction (MI). A tightly regulated restriction of their persistence within the damaged heart, however, is required to avoid nonspecific tissue degradation. Here, we sought to profile the direct contribution of Reg3beta, a novel cardiomyocyte–derived cytokine, in controlling the maintenance of Nphs at sites of cardiac injury.

METHODS: We employed immunohistochemical analysis combined with flow cytometry to evaluate direct interactions of Reg3beta with Nphs in infarcted hearts of wildtype (WT) mice. Dynamic ratios of viable and dead Nphs were profiled in WT and Reg3beta deficient (Reg3b–/–) mice by flow cytometry post–MI. To this end, we studied direct cytotoxic effects of Reg3beta by using functional in vitro assays.

RESULTS: We observed a specific binding of Reg3beta to Nphs within the interstitial space of infarcted hearts of WT mice. Flow cytometry based identification of Reg3beta–positive Nphs yielded two separate Nph populations, of which Reg3beta–high Nphs featured a decreased viability in contrast to Reg3beta–low Nphs. Importantly, the proportion of dying Reg3beta–positive Nphs increased continuously within the first 7 days after the onset of MI and thereby coincided with a decreased rate of cellular death of Nphs in Reg3b–/– mice at day 4 and 7 post–MI. Treatment of cultured Nphs with Reg3beta finally provided evidence for a direct cytotoxic effect of Reg3beta on Nphs.

CONCLUSION: The release of Reg3beta by cardiomyocytes and its binding to Nphs within the ischemic heart implies an endogenous regulatory loop to propagate the removal of Nphs in a temporally and spatially defined manner.

P127 GENERATION OF EXTRACELLULAR VESICLES AND PHAGOCYTOSIS: TWO FUNCTIONS, ONE RECEPTOR, TWO DIFFERENT SIGNALING PATHWAYS

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Generation of EVs enriched in granule proteins and possessing antibacterial effect (aEV) depends on opsonin receptors which are also involved in phagocytosis. Our goal was to investigate the interrelation of the two processes. The specific question was whether identical receptors are involved and whether phagocytosis is needed for aEV formation.

Medium-sized EVs were obtained in two centrifugation steps and filtration from neutrophils (PMN) isolated from human peripheral blood or murine bone marrow. Murine PMN-EVs were characterized in detail using dynamic light scattering and electron microscopy. EVs were quantitated by flow cytometry and protein determination. Phagocytosis of opsonized particles was followed by flow cytometry and verified by confocal microscopy.

In human PMN aEV formation critically depended on stimulation of the integrin Mac-1/CR3, whereas Fc receptors (FcR) did not seem to be involved. Mac-1 signaling also affected cargo sorting in aEVs. In contrast, in phagocytosis FcR had a definitive role and maximal rate was achieved by combined FcR and Mac-1/CR3 stimulation. Importantly, aEV production could be initiated also on adhesive surface where phagocytosis was not possible. In genetically modified animals we observed significant divergence in aEV generation and phagocytosis: deletion of Src kinases impaired phagocytosis but did not affect aEV generation, whereas deletion of phospholipase Cγ2 or calcium depletion prevented aEV generation but did not influence phagocytosis.

In conclusion, aEV production and phagocytosis are independent processes, Mac-1/CR3 plays central role in both cellular reactions, but they are organized via different signaling pathways.

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P128 BLOCKADE OF PAR2 INHIBITS NEUTROPHIL RECRUITMENT AND ATTENUATES ALLERGEN-INDUCED ACUTE LUNG INFLAMMATION IN MICE

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Proteinase-activated receptors (PARs) are G-protein-coupled receptors comprising a family of four members, and PAR2 has been implicated in mediating allergic airway inflammation. We have demonstrated that PAR2 plays a role in eosinophil (Eo) recruitment in experimental allergen-induced pleurisy supporting additional evidences for a role for PAR2 in allergic diseases. Objective: Study the effects of PAR2 blockade on the neutrophil recruitment and on allergen-induced acute lung inflammation. Methods: Ovalbumin (OVA)-sensitized BALB/c mice were systemically pretreated or not with selective PAR2 antagonist ENMD1068 (ENDMD, 0.5 mg/Kg) 1h before OVA (10μg) or PBS intranasal instillation and bronchoalveolar lavage fluid obtained 30 min to 2 h after, to evaluate neutrophil infiltrating, cytokine levels and PAR2 expression. Lungs were removed 2 h after OVA challenging to perform vascular lung permeability measured by spectrophotometric absorbance. Statistical analyses: One-Way ANOVA followed by Newman-Keuls post-test (UFMG Ethics Committee for Animal Use, certificated number 348/2014). Results: ENMD inhibited Neo recruitment, decreased PAR2 expression, levels of CXCL1, CCL5, IL-6 and extravasation of Evans blue to the lung when compared to OVA-treated mice. Furthermore, ENMD significantly increased IL-10 levels 30 min and 2h after immunogen instillation. Conclusion: This study suggests a role for PAR2 on neutrophil recruitment in allergen-induced acute lung inflammation, at least in part through the cytokine modulation at the allergic inflammatory site. Financial Support: FAPEMIG (PPM-X).

P129 A METHOD FOR CRYOPRESERVATION AND RECOVERY OF VIABLE HUMAN NEUTROPHILS.

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Objective: The dynamic role of neutrophils that allows quick and continuous response to potential threats makes it difficult to perform experiments and assays, which must be uninterrupted. Such limitation reduces the possibilities of multi-site experiments and therefore, of discovery. This work aimed to develop a simple method of neutrophil cryopreservation at 80°C that preserves morphology, viability and cell functions. Methods: A cryoprotectant solution of 35,8% glycerol, Hanks balanced salt solution and 5% autologous serum was added to the neutrophil pellet, immediately after density gradient isolation. After glycerolization for 10 minutes at 0ºC, the system was incubated for 10min at -20°C before being stored at -80°C. Thawing was performed in steps at -20°C and 0°C and the glycerol concentration was gradually decreased by dilution steps. Aliquots were taken for morphology analysis and viability testing. Cryopreserved and fresh purified neutrophils were tested for chemotaxis by Boyden chamber assay, activation by fMLP, reactive oxygen species production though the NBT test and phagocytosis by incubation with Saccharomyces cerevisiae. Results: The mean neutrophil count was 1,94 x 1E7 before freezing and 1,45 x 1E7 after thawing with cell survival rates of 98% and 89% respectively and a mean loss of 25%. Normal morphology with minor injury to the membrane was confirmed by microscopy analysis. NBT test, yeast phagocytosis and chemotaxis tests showed a similar activation pattern for cryopreserved cells compared to fresh controls, with no statistical difference. Conclusion: Our study describes a simple protocol for neutrophil cryopreservation at -80° with satisfactory cell count, viability and normal rates of chemotaxis, ROS production and phagocytosis.

P130 *Pseudomonas aeruginosa* EVASION OF NEUTROPHIL FUNCTIONS AND BACTERIAL CLEARANCE IN EARLY CYSTIC FIBROSIS LUNG INFECTION

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Failure to clear Pseudomonas aeruginosa (PA) airway infections by innate host defenses during early stages leads to chronic infection in the lung of Cystic Fibrosis (CF) patients. Since the adequate antibacterial functions of neutrophils (Nϕ) are necessary for successful PA eradication, we therefore hypothesized that PA that are not eradicated at the time of early infection and persist in CF patients elicit impaired Nϕ functions. We compared in vitro Nϕ phagocytosis and intracellular bacterial killing in response to persistent (persisters) vs eradicated (eradicaters) PA clinical isolates from the Sick Kids PA eradication clinical study. A lower phagocytosis and intracellular killing of PA were observed in persisters compared to eradicaters. This suggests that persisters exploit strain specific bacterial factors to evade Nϕ responses. Associations between bacterial factor (type IV pilus mediated twitching motility, flagellum mediated swimming motility, alginate (mucoidy), and Psl), and Nϕ phagocytosis were evaluated. We observed that twitching motility was only modestly correlated with Nϕ phagocytosis, but not swimming motility. Among all PA isolates, mucoid ones are more prevalent in persisters compared to eradicaters. Psl expressing PA isolates are also more prevalent in persisters and Psl maybe an important mechanism that contributes to defective Nϕ responses in persisters, particularly non-mucoid ones. These results highlight the potential role in specific PA and Nϕ interactions that contribute to bacterial eradication in CF patients.

P131 A CHOKEHOLD OF SOCS PROTEINS UPON STAT FACTORS IN HUMAN NEUTROPHILS

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Cytokines and chemokines produced by neutrophils are key mediators of inflammation and immunity, and we have shown that many are regulated by discrete transcription factors (e.g. NF-κB, CREB, C/EBP). The STAT transcription factors are also present and inducible in neutrophils, and it is widely presumed that they control cytokine production as they do in other leukocytes. Using selective inhibitors and specific siRNAs, we show that STATs control the induction of many genes in response to G-CSF, GM-CSF and IFNγ. Amongst these genes, some encode cytokines (IL-1β, CXCL1, CXCL10) or membrane receptors (CD64, PD-L1, TLR5, IL-18R). Intriguingly, the inhibition or absence of STATs did not affect the secretion of these cytokines or the surface expression of these receptors. To understand this contradiction we investigated the role of a family of STAT repressors, the SOCS proteins. We found that G-CSF, GM-CSF and IFNγ induced the gene expression of many SOCS (CIS, SOCS1, SOCS3) in a STAT-dependent manner. Using siRNAs we depleted SOCS mRNA levels. This prolonged STAT1, 3 and 5 tyrosine phosphorylation, indicating a repressor effect of SOCS on STAT signaling in neutrophils. In keeping with this interpretation, the absence of SOCS drastically augmented both the gene expression and secretion of cytokines such as CXCL10 and IL-12. The gene and surface expression of membrane receptors (CD64, PD-L1) was similarly affected. Conversely, overexpression of SOCS1 or SOCS3 in neutrophilic PLB-985 cells had the opposite effect. In summary, while STAT factors do control the expression of several target genes in neutrophils, they seem to have little or no effect on functional responses in these cells, as their quick and strong repression by SOCS proteins seems sufficient to prevent durable induction of STAT-dependent genes.

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