C1. Inflammation and cancer: intracellular signaling and beyond

Lectures

L1.1

ADAM17 orchestrates pro- and anti-inflammatory cytokine activities in inflammation and cancer

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Cytokine receptors exist in membrane bound and soluble form. While most soluble receptors are antagonists, some soluble receptors are agonists like soluble receptors of the gp130 cytokine family. *In vivo*, the IL-6/soluble IL-6R complex stimulates several types of target cells not stimulated by IL-6 alone, since they do not express the membrane bound IL-6R. This process has been named trans-signaling [1].
We have shown that soluble gp130 is the natural inhibitor of IL-6/soluble IL-6R complex responses. The recombinant soluble gp130 protein is a molecular tool to discriminate between gp130 responses *via* membrane bound and soluble IL-6R responses. We have constructed a fusion of soluble gp130 and the Fc portion of human IgG1. This sgp130Fc protein proved to be efficient in blocking responses via the IL-6/soluble IL-6R complex without affecting IL-6 responses, which are mediated *via* the membrane bound IL-6R [1]. The soluble IL-6R is mostly generated by proteolysis of the IL-6R transmembrane protein. Shedding of the IL-6R is mediated by the metalloprotease ADAM17, which is also responsible for the cleavage of TNFα and ligands of the EGF-R. We generated hypomorphic ADAM17 mice, which have undetectable ADAM17 protein levels in all tissues but which are still viable. Using these mice in different inflammation models we could show that activation of ADAM17 has different effects on the activation of the immune response as well as on induction of regenerative responses [2, 3]. Therefore, ADAM17 is a molecular switch of inflammatory and regenerative responses of the body to stress [3].
Using the sgp130Fc protein or sgp130Fc transgenic mice we further demonstrate that in several chronic inflammatory diseases and cancers including inflammatory bowel disease, peritonitis, rheumatoid arthritis, colon cancer, ovarian cancer and pancreatic cancer, IL-6 trans-signaling *via* the soluble IL-6R is a crucial step in the development and the progression of the disease. Therefore, sgp130Fc is a promising novel therapeutic agent for the treatment of chronic inflammatory diseases and cancer [1, 4-6].

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L1.2

Osteopontin: a multifunctional signaling protein at the crossroads of inflammation and cancer

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Osteopontin (OPN, Spp1), a secreted glycoprotein, interacts with integrin and CD44 receptors, and regulates many cellular functions such as adhesion and migration. It has multiple immunological functions and is secreted by activated macrophages, leukocytes and activated T lymphocytes. OPN is up-regulated at sites of inflammation and its involvement in inflammation, autoimmune disorders and cancer has been demonstrated. Expression of OPN is up-regulated in tumor tissues compared to normal tissues and correlates with malignancy grade in several types of tumors, including gliomas. Our *in vitro* studies revealed that glioma cells secrete soluble factors which convert microglial cells (brain macrophages) into amoeboid cells supporting glioma invasiveness, while attenuating inflammatory responses. Proteomic analysis of glioma-conditioned medium revealed that one of such factors is a metastasis-associated, integrin-binding, — osteopontin. Interference with OPN binding to integrins or gene silencing in glioma cells, abolished microglial activation and its pro-invasive influence on glioma invasiveness in cultured cells. Depletion of OPN in glioma cells impaired gliomas growth in mice and reduced microglial activation. Malignant gliomas can originate from neural progenitors or cancer stem cells (CSC), which acquired mutations in tumour suppressors or in signal transduction pathways or both. Glioma SC can suppress innate and adaptive immunity. They are characterized by the high activity of ABC transporters, expression of cell surface markers and pluripotency transcription factors: Oct3/4 and Nanog. Three methods for isolation of glioma SC have been employed: 1/Rhodamine 123 (Rhod123) exclusion assay followed by FACS sorting for Rhod123 negative and positive subpopulations; 2/selection with anti-EGFR antibody for the EGFR negative cell subset enriched in CSC; 3/functional sphere-forming assay based on stem cell ability to grow as unattached spheres. We demonstrate that cells from EGFR(-), Rhod(-) fractions and anchorage-independent spheres overexpressed *Nanog* and Oct3/4, confirming enrichment of those cell populations for CSC. The same cell subpopulations expressed much higher levels of OPN mRNA than their counterparts. The elevated expression of OPN in glioma stem cells and its ability to activate macrophages without inducing immune response suggests a novel role of OPN in glioma pathology.

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L1.3

Cancer-associated inflammation — questions of molecular marker specificity

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Screening, diagnosis, prediction of treatment response and monitoring of cancer patients requires reliable cancer biomarkers. With the rapid development of new technologies, thousands of potential cancer biomarkers have emerged but only a few have found their way to clinical application.

Accumulating evidence shows that not only local but also systemic inflammatory reactions are implicated in cancer and interfere with the molecular image of the disease. We have shown that many of the so-called tumour markers are inducible in normal peripheral blood mononuclear cells.

The difficulty in tumour biomarker research lies in determining which markers are specifically linked to cancer, and do not just reflect unspecific secondary changes or accompanying diseases. Thus, a reliable control to differentiate cancer-specific changes is of utmost importance, especially when employing molecular technologies, highly sensitive but, as such, presenting significantly decreased specificity. Unfortunately, most modern biomarker studies, such as those employing proteomics, circulating tumour cell and free nucleic acid assessments, epigenetics and metabonomics, seem to ignore inflammation as an inherent component of cancer disease, and examine cancer patients' samples against those of healthy, inflammation-free persons. Then, the prognostic value of differentially expressed markers is often regarded as an indirect proof of their cancer specificity, but it is the systemic inflammatory response in cancer patients that frequently relates to clinical parameters, and mere measurements of the inflammatory factors present prognostic value.

It should be strongly emphasised that the difference in the expression of a marker between cancer and normal samples does not provide grounds for firm conclusions on cancer specificity of the marker. All potential cancer biomarkers should be validated against their expression in inflammatory conditions, and their independent predictive value should be examined in the context of unspecific parameters of systemic inflammation. Otherwise we will end up using advanced technologies to assess the inflammatory reactions in cancer patients.

L1.4

Regulation of NFkappaB activity

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The ubiquitous transcription factor NFkappaB (NF-κB) is a key mediator of the immune, inflammatory and stress responses, as well as the main regulator of development and differentiation, cellular growth and apoptosis. The NF-κB complex is activated by numerous stimuli and it is present in cytoplasm in the inactive form complexed with its inhibitor, IkappaB. Stimulation of the cell leads to IkappaB phosphorylation followed by degradation in proteasome-dependent pathway. This results in NF-κB translocation to the nucleus and induction of gene expression. It is known that NF-κB is persistently active in a number of disease states, including cancer, arthritis, chronic inflammation, asthma or neurodegenerative disorders. Constitutive NF-κB activity has been implicated in the malignant progression of numerous hematologic and solid tisssue malignancies with certain reports indicating that NF-κB activation correlates with primary tumor size. Regulation of activity of NF-κB is tightly controlled by positive and negative mediators operating at various levels of cell and tissue organisation. We found recently that one of the negative regulators of NF-κB activity is a novel protein, MCPIP, induced by a potent chemokine, MCP-1 (macrophage chemoattractant protein). MCPIP can be regarded as a multidomain and multifunctional protein, regulating inflammatory reactions on various levels. We (Mizgalska *et al.,* 2009, *FEBS J* **276**: 7386-7399) and Matsushita *et al.* (2009, *Nature* **458**: 1185-1190) demonstrated that MCPIP affects the stability of mRNA coding for proinflammatory cytokines: IL-1, IL-6 and IL-12. Its characteristic RNase properties depend on the presence of a PIN domain. Moreover, we found (Skalniak *et al.*, 2009, *FEBS J.* **276:** 5892-5905) that MCPIP directly decreases NF-κB binding activity in cells stimulated with LPS or IL-1. Our recent studies indicate that MCPIP interacts with proteins present in the signaling pathway of NF-κB: TANK, TRAF2 and TRAF6, but its influence on NF-κB activity is triggered by a mechanism not yet fully understood. We also found that MCPIP plays a significant role in cell differentiation, proliferation and apoptosis and thus MCPIP might become soon an important target in the development of innovative treatment of diseases with inflammatory background.

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Oral presentations

O1.1

Does the lack of GRHL1 activity increase the chance of skin cancer development?

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Grainyhead-like (GRHL) family consists of highly conserved homologs of *Drosophila* Grainyhead (GRH) transcription factor. In mice, GRHL3 maintains the integrity of skin barrier formation through the regulation of transglutaminase 1. In *Drosophila melanogaster*, GRH controls the expression of DOPA decarboxylase which serves analogous function preserving the integrity of fly cuticle. Given the conserved role of GRHL in maintaining epithelial integrity, it is noteworthy that the evolutionary origin of GRHL family appears coincident with evolutionary origin of the epithelium. GRHL1 is normally active in the developing epidermis and hair follicles. Mice lacking this factor display an abnormal hair coat (defective hair anchoring) and reduced expression of desmoglein 1, a member of the desmosomal cadherin family and a direct target of GRHL1 regulation.
It is still uncertain whether GRHL1 is involved in skin cancer development induced by UV radiation and what is the role of GRHL1 in skin barrier formation. The main goal of my research is to investigate skin cancer formation in *Grhl1*-deficient mice upon exposure to UV radiation. Experiments are performed on mice with three different G*rhl1* genotypes: wild type, heterozygous and null. *Grhl1* knock-out mice were generated in the Royal Melbourne Hospital (Australia) using genetic engineering methods. These animals were provided to us under a Material Transfer Agreement.
Consensus DNA binding sequence of GRHL1 transcription factor is already known, which makes it possible to predict its putative targets genes that are linked to skin cancer development. My research is likely to discover novel signal transduction pathways that are relevant to skin cancer formation. I will utilize various molecular biology, histological and bioinformatic methods.

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O1.2

Loss of the tumor suppressor CYLD causes enhanced NF-kappaB and Wnt/β-catenin signaling in multiple myeloma

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In multiple myeloma, the Wnt/β-catenin pathway and NF-kappaB pathway are frequently aberrantly activated, leading to increased tumor proliferation, survival and dissemination. The deubiquitinating enzyme *CYLD* was originally identified as a tumor suppressor that is mutated in familial cylindromatosis. CYLD is a key negative regulator of NF-kappaB signaling which acts by deubiquitinating tumor necrosis factor (TNF) receptor-associated factor (TRAF2), TRAF6, and NEMO (NF-kappaB essential modulator, also known as IkappaB kinase gamma). It was recently demonstrated that CYLD acts also as a negative regulator of Wnt/β-catenin signaling through a mechanism in which hyperubiquitination of polymerized Dvl drives enhanced Wnt responses. Interestingly, in MM, deletion and missense mutations of *CYLD* have been reported. Here, we show that *CYLD* expression is frequently lost in MM tumors and it is strongly correlated with a proliferative gene-expression profile. Functional assays with inducible knockdown of *CYLD* in MM cells revealed that *CYLD* silencing increases autocrine and Wnt3a stimulated Wnt signaling and dramatically enhances the NF-kappaB responsiveness of MM cells. Consequently, an increase in survival and proliferation of malignant plasma cells with *CYLD* knockdown was observed. These findings identify loss of *CYLD* expression as a potential cause of aberrant Wnt and NF-kappaB pathway activation in MM, enhancing proliferation, survival and dissemination of malignant cells.

O1.3

Heme oxygenase-1 as possible therapeutic target in treatment of rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is one of the commonest forms of soft tissue sarcomas in children and young adults and is usually derived from primitive mesenchymal cells. Two main groups of RMS may be mentioned- embryonal (eRMS) and alveolar (aRMS) subtypes of RMS. aRMS is characterized by higher metastatic potential and malignancy. Those types of tumor share abrogation of PAX regulatory pathway followed by improper action of downstream elements, including MyoD and muscle specific microRNAs (myo-miRNAs) miRs 1, 133a, 133b and 206. microRNAs are novel class of particles involved in regulation of gene expression and myo-miRNAs are known to promote myocyte differentiation.

We have shown previously that heme oxygenase-1 (HO-1), which is key anti- oxidant enzyme within the cell can act as inhibitor of muscle differentiation. HO-1 action is possibly mediated by stromal cell derived factor 1 (SDF1), p38 kinase pathway and inhibition of MyoD and muscle specific microRNAs (myo-miRNAs).

Given its effect in muscle differentiation, we speculated that HO-1 can also affect invasiveness of RMS. Indeed, according to the data obtained from *in vitro* experiments on 6 RMS cell lines (2 of embryonal and 4 of alveolar type) aRMS are characterized by higher expression of HO-1. It also correlates with lower levels of myo-miRNAs, SDF1, cMET and possibly with tumor malignancy. Interestingly silencing of HO-1 using specific siRNA leads to reversal of effect of this protein on SDF1, cMET and myo- miRs expression

Recently, many studies focused on cancer have demonstrated the great potential of the genomic approach based on tumor expression profiles. As up-to-date common treatment of RMS involves aggressive chemo- and radiotherapy it is crucial to identify possible molecular targets for aimed therapy. Here we indicate that HO-1 can be one of proteins of great interest in this field of study.

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O1.4

Tumour-derived microvesicles contain interleukin-8 and modulate production of chemokines by human monocytes

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Interaction of tumour infiltrating macrophages (TIM) with cancer cells leads to the production of both pro- and anti-tumour mediators by TIM. Contact of monocytes/macrophages with tumour cells *in vitro* induces production by the former of TNF, IL-10, IL-12, reactive nitrogen and oxygen intermediates as well as chemokines. Tumour cells, like many other cells, are shedding small, vesicular structures called microvesicles (MV). Tumour-derived MV (TMV) may be an important mode of communication between the cells. The present study was designed to determine whether TMV may modulate chemokine production/secretion by human monocytes as circulating precursors of TIM. TMV induced secretion of CXCL8 and CCL2, 3, 4, 5 chemokines and accumulation of their mRNA in monocytes. Moreover, TMV enhanced angiogenesis in NOD-SCID mice by delivering chemokines and *via* stimulation of monocytes. In addition, TMV may be storage for chemokines (mRNA and protein) thus inducing chemotaxis of blood leukocytes. In conclusion, TMV interact with monocytes and alter their biological activity. This implicates the novel mechanism by which TIM may be affected by tumour cell not only by a direct cell to cell contact, soluble mediators, but also by TMV.

Posters

P1.1

Enhanced neutrophil adhesion to endothelium is modulated by kinins and their des-Arg metabolites

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The adhesion of neutrophils to vascular endothelium initiates their infiltration into tissues where these polymorphonuclear leukocytes (PMN) secrete numerous mediators that regulate the inflammatory state. On the other hand, bioactive bradykinin-related peptides (kinins) are abundantly expressed at inflammatory sites and in tumors. In contrast to a well documented role of these peptides in PMN chemotaxis and recruitment, their influence on neutrophil adhesion to vascular walls is still not well characterized. In this work we studied the effect of bradykinin (BK) and des-Arg^{10}-kallidin (DAKD) on PMN adhesion to extracellular matrix proteins such as fibrinogen and fibronectin as well as to the human microvascular endothelial cell line HMEC-1.
Both BK and DAKD caused an enhancement of PMN adhesion. After pre-incubation of PMN with BK or DAKD, the PMN adhesion to microplate-immobilized proteins increased by 30% as compared to non-stimulated PMN. This effect was even stronger when PMN adherence to HMEC-1 cells was studied. In the latter case, the enhancement of PMN adhesion was higher under influence of DAKD than that in the presence of BK (by 100% and 50%, respectively, as compared to non-stimulated PMN). The BK-induced neutrophil adhesion to HMEC-1 was significantly enhanced when leukocytes or endothelial cells were additionally treated with interleukin 1β (IL1β). However, this effect was attenuated by an inhibitor of carboxypeptidase M, the enzyme involved in the production of des-Arg kinins, suggesting an essential role of these kinin metabolites in the leukocyte adhesion. In this study, we also demonstrated that BK and DAKD caused changes in the expression of Mac-1 integrin subunit CD11b. The CD11b expression increased by 10%, 17% and 20% for BK, DAKD and BK/IL1β stimulation, respectively, as compared to non-stimulated cells. Likewise, the expression of the other Mac-1 subunit CD18 on the cell surface also increased after stimulation and this effect was somewhat larger when the cells were treated with BK (about 16% and 40% above non-stimulated cell expression for BK and BK/IL1β stimulated cells, respectively). Additionally, BK induced a significant expression of ICAM-1 adhesion molecule in HMEC-1.
The data presented in this report provide an evidence for the regulatory effects of kinins on PMN adhesion to endothelial wall and describe new aspects of the leukocyte infiltration into inflamed tissues, especially in a context of cancer-forming processes.

P1.2

Organo-selenium compound Selol attenuates pro-inflammatory response in murine macrophages induced by LPS

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Selol is a mixture of selenitriglicerides synthesized from sunflower oil. As Selol contains the element selenium (Se) in its structure, it is suspected to exhibit chemopreventive and anticancer activity. Selenium is a trace element necessary for the regular functioning of living organism, e.g. is required for the optimal functioning of the immuno system. In macrophages Se acts as a major antioxidant in the form of selenoproteins to mitigate the cytotoxic effects of reactive oxygen species. Se exists in several forms: elemental selenium (0), selenide (-2), selenite (+4), and selenate (+6). The toxicity of the different forms of selenium depends on the oxidation state of Se. The highest biological activity as an antioxidant and anticancer agent is assigned to selenium compounds containing tetravalent Se (+4). Selol contains Se (+4). Organo-selenium compounds with a Se at the +4 oxidation state have the highest activity as free radical scavenger and anticancer agents.
The aim of the study was to test the effect of Selol 5% on some parameters of the signal transduction pathway induced in macrophages by bacterial lipopolisaccharide endotoxin (LPS). Cell line of murine macrophages RAW 264.7 was used. Cells exposed to Selol showed an increased production of reactive oxygen species in the test with dihydrorhodamine (DHR 123). Moreover, Selol restored the viability of LPS-treated cells evaluated by trypan blue exclusion assay. Selol decreased LPS-induced expression of pro-inflammatory inducible nitric oxide synthase (iNOS), with concomitant decrease of nitric oxide (NO) level in macrophages incubated together with LPS and Selol.
Our results indicate anti-inflammatory properties of Selol 5%.

P1.3

The influence of Selol on human fibroblast cells

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The research of potential anti-cancer medicines is directed to showing their strong destructive effect on the pathologically altered cells, causing the least damage to the healthy tissues at the same time. One of the potential anti-cancer medicines is Selol, a mixture of selenitetriglycerides containing selenium on the +4 level of oxidation (Patent, Pol. PL 176530 (Cl.A61K31/095)).

In the previous research carried out by our team, we received cytotoxic influence of Selol on HeLa cancer cells as well as significant changes occurring in gene expression, connected with the response to oxidative stress. The aim of the following study was to test the influence of Selol on normal human fibroblasts (BJ). The specification of the changes in gene expression was preceded by the assessment of the effect of Selol (at the concentration range of 12.5–750 μM Se) on the survival of normal cells BJ (MTT) and the induction of apoptosis (annexin V/IP test). In the research we used sodium selenite (at the concentration range of 1–20 μM Se) as the reference compound.

No significant influence of Selol on the proliferation of BJ cells or its apoptotic and necrotic effect was proved. For sodium selenite, we received a dose-dependent influence on the proliferation and induction of apoptosis and necrosis of the tested cells.

While testing gene expression in BJ cells, we observed a strong activity excitement of many oxidative stress genes, already for a lower concentration of Selol (37.5 μM Se). Together with the increase of Selol concentration (75 μM Se), a further increase in the expression of those genes followed. From the comparison of responses to selenium penetrating from both compounds, it is due that sodium selenite acts more prooxidatively, with more poorly expressed response from the genes involved in the defence against stress. All the received results confirm a much lower toxicity of the tested compound of selenium for BJ normal cells than of the reference compound — sodium selenite.

P1.4

$CD4^+CD25^{high}$ regulatory T cells frequency in peripheral blood of gastric cancer patients during radiotherapy

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Gastric cancer is often diagnosed at advanced stages, patients have regional node involvement and/or extension of tumor through the stomach wall, or into the adjacent organs. Preoperative radiotherapy may downstage the tumor and potentially increase the rate of resectability, but it causes inflammation and immune imbalance. Furthermore, the disorder of immune homeostasis may influence the effectiveness of radiotherapy and impede tissue repair.

Treg cells play an active and significant role in the progression of cancer, and have an important role in suppressing tumor-specific immunity. Regulatory T cells, characterized by coexpression of CD4 and CD25 markers, can inhibit the immune response mediated by Tc and Th cells. $CD4^+CD25^{high}$ cells have been demonstrated to suppress various types of immune responses, including autoimmune, antimicrobial and antitumor immune responses by inhibiting T, B and NK cells. They down-regulated the activity of effector function against tumors, resulting in T cell dysfunction in cancer-bearing host.

We previously showed increased populations of $CD4^+CD25^{high}$ cells in peripheral blood T cells in patients with gastric cancer in comparison with healthy donors. The current study was designed to investigate the changes in peripheral $CD4^+CD25^+$ regulatory cells in cancer patients and the influence of different radiotherapy dose on immunity function.

The phenotypes of lymphocytes were analyzed in peripheral blood in patient with M0 and M1 gastric cancer. None of the patients received surgery, radiotherapy, chemotherapy, or other medical interventions before this study. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Sigma Aldrich, USA) density gradient. Cell surface marker analysis was performed using fluorochrome labeled mouse anti-human monoclonal antibodies targeted against CD45 FITC, CD3 PE, CD4 PerCP, CD25 APC together with appropriate isotype controls to allow identification of positive and negative cell populations. Phenothypes and proportions of each subpopulation were analyzed with FACSCanto flow cytometer.

We investigated the changes of Treg cells in peripheral blood lymphocytes in patients undergoing radiotherapy in comparison with reduction in the absolute number of T cells and changing of other lymphocyte subsets.

A better understanding of the underlying mechanism of Treg regulation or of the strategy for controlling this lymphocytes may lead to more effective anticancer therapy.