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REPORT OF MEETING

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Organizers: **L Canesi, T Balbi, M Auguste, E Grasselli, L Vergani, I Demori, R Fabbri, M Montagna, A Voci**

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Session 1. Chairmen: Laura Canesi, University of Genoa, Genoa, Italy and Giuseppe Scapigliati, University of Tuscia, Viterbo, Italy
Nanoparticles and the immune system

Evolution of innate immunity, lessons learned for assessing safety and efficacy of nanomaterials and nanodrugs

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Innate immune defensive mechanisms are the major surveillance and effector mechanisms deputed to maintaining the integrity and functionality of a living organisms. From plants to human beings, innate immunity has conserved a number of defensive cells, molecules and pathways. Innate immunity is the only type of immunity present in plants and invertebrates, at variance with higher vertebrates that also display slower but more specific adaptive immune functions. Immunological memory, a feature though to characterise adaptive immunity, is clearly present in innate immunity although based on different mechanisms, and can be clearly displayed by phagocytic cells, which are rapid and efficient cells in recognising and destroying invading microorganisms or foreign particles. We have studied both innate immune responses and the development of innate memory in human blood monocytes *in vitro* in comparison with the marine tunicate *Ciona intestinalis*, in which adaptive immunity is absent. The strong primary response to a bacterial challenge (*e.g.*, LPS) can prime cells *in vitro* or animals *in vivo* to respond differently to a subsequent challenge with the same or with a different stimulus. Human cells primed with LPS showed a significant modulation of their activation capacity in response to a second challenge (with LPS or other stimuli), with a clear decrease in the capacity of producing TNFα. On the other hand, challenge with LPS strongly increased the phagocytic ability of *C. intestinalis* haemocytes primed with either LPS or LTA, whereas challenge with LTA decreased it. For human cells, strong donor-to-donor variations were evident, suggesting that previous *in vivo* exposure to diverse external agents may change the reactivity of blood monocytes to *in vitro* stimuli. Although significant quantitative inter-individual differences were noted, in *C. intestinalis* the development of innate memory was more reproducible in terms of increase or decrease of secondary response, suggesting a limited or similar influence of the previous life conditions.

The comparative study of innate memory is expected to generate information that will help us controlling and modulating memory development in

future personalised immunoprophylactic and immunotherapeutic approaches.

Human immune cells as target of Gold NPs (AuNPs): potential impacts on immune responses

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Gold Nanoparticles (AuNPs) are becoming every day more part of our life. Because of their unique optical and physical properties, their applications in different fields are dramatically increasing. Particularly in medicine, they have been suggested as new delivery systems, diagnostic tools, vaccination adjuvants, as enhancer for radiation therapy and as novel contrast agents for Computer Tomography (CT).

Some of these approaches include high doses of intravenously and sometimes repetitively injected Gold NPs which then can spread throughout the body coming in contact with many different cell types including immune cells.

Since this is a branch of science in active development and still little is known about the potential effects of NPs on the immune system, especially in the context of long term and chronic exposure with concomitant pathologies, there is an urgent need to better understand the possible functions of AuNPs on immune cells with phagocytic properties such as macrophages and dendritic cells (DCs).

A matter of interest is also the extremely reactive surface of NPs, because NPs in fact are capable of binding proteins and molecules, changing their conformation and maybe, their activity.

In order to identify common mechanisms/markers of cellular reactivity that can be predictive for determining risk or safety of these nano-objects, the current study investigates the role of AuNPs of different sizes and surface area, on modulating dendritic cell-activation induced by LPS.

We observed that treatment of human monocyte-derived dendritic cells with LPS in combination with endotoxin-free 26nm-AuNPs, alters the expression of specific cell surface markers and modulates the release of certain cytokines. No similar effects were observed with AuNPs with a size of 4, 9 and 11 nm. This suggests that 26 nm AuNPs may have specific properties, maybe in terms of surface area, that contribute to their immune modulatory properties. Our observation could also substantiate some earlier evidences that the medical use of colloidal gold might be beneficial to reduce the swelling and inflammation of joints in patients with rheumatoid arthritis.

Optimization of a cryopreserved immune cell product for nanosafety testing

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Although nanoparticles (NPs) have always been on our planet, recently the impact of nanomaterials on health and the environment has received considerable attention. NPs are defined as materials within nanoscale dimensions and nowadays NPs are applied in various fields such as chemistry, electronics, consumer goods and medicine.

There is now a pressing need for more predictive and efficient methodologies to examine nanoparticle immunosafety on humans, animals and the environment. Therefore, the purpose of this project was to set up a panel of cell-based assays to measure the human innate inflammatory response to NPs under analytical conditions and in formats representing industrial prototypes for commercial exploitation.

The research objective was to establish and optimise a ready for use immune product in 96 well plate format, that was translatable to a 3D assay format, and which could assess the activation of immune cells after exposure to different kinds of NP. The developed “plug and play” immune-Cryotix™ format is based on the activation of human monocyte derived macrophages, which are essential as the first line of defence in many organisms, and the detection of cytokines secreted by these macrophages after inflammatory stimulation.

Briefly, peripheral blood mononuclear cells (PBMC) were isolated from samples of whole blood by immuno-magnetic selection. Monocytes were then differentiated to M1 and M2 macrophages, the provenance of which was confirmed via flow cytometric analysis, and optimised cryopreservation protocols were established. Cryopreserved plates can be stored at -150 °C, shipped to the customer via ACS shipping container and thawed 24 h before the inflammatory screen is performed.

Recovered cryopreserved macrophages maintain high viability and display comparable morphology to unfrozen macrophages. Inflammatory cytokine secretion, in unstimulated or LPS-stimulated macrophages was similar for control and cryopreserved cells. Experiments are ongoing to improve the performance of the immune-Cryotix® product and also to establish standardise assay readouts for NP testing.

Combining whole-organism toxicity endpoints, stress-related enzyme levels, and cellular & humoral immune system markers of *Porcellio scaber* to evaluate nanoparticle toxicity, using gold nanoparticles as an example

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Due to their stability and ease with which they can be synthesised, gold nanoparticles are popular nanoparticles (NPs) in nano-bio interaction research. With the myriad of potential uses they

could have, it is inevitable that these NPs will eventually reach the environment and therefore it is imperative that the possible side effects they may have be evaluated.

It is thought that organisms may modulate their immune system after exposure to NPs as they may recognise NPs as foreign in much the same way they would recognise and respond to invading bacteria. The aim of this study was to investigate whether ingestion of gold NPs alters the immune response of the terrestrial isopod, *Porcellio scaber*, and how these changes will be reflected at higher levels of organisation such as feeding and mortality. As the immune system is an early responder to foreign matter, studying it in conjunction with traditionally used parameters of toxicology can give more information into the possible effects these particles may have.

Animals were fed gold NPs for 14 days, during which time the feeding, defecation and survival rates of the animals were recorded, these are the traditional environmental toxicity endpoints. After 14 days, the hemolymph was removed and the number, viability and proportion of hemocytes were counted. Along with cellular tests, the humoral side of the immune system was investigated by measuring the activity of the enzyme phenoloxidase in hemolymph, which is associated with melanisation. The levels of stress markers, glutathione S-transferase (GST) and soluble acetylcholinesterase (AChE), were also assayed. The removal of hemolymph has no impact on the other measurements, meaning no extra animals have to be used while garnering this immunological information. Animals injected with a non-lethal dose of LPS acted as a positive control for the activated immune system.

Results so far indicate that the gold NPs tested had no significant effect on the feeding, mortality or defecation rates of isopods. There were also no significant changes to the cellular immune system and preliminary results suggest no effects on the humoral immune system. These results suggest that gold NPs at the selected, environmentally relevant exposure concentration are not toxic nor immunomodulating agents to *P. scaber*. We discuss the immune system as an additional endpoint in assessing the effects of nanomaterials on environmentally relevant organisms.

"Les liaisons dangereuses": do nanoparticles affect immune defense by modulating innate memory?

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Innate immunity is evolutionarily conserved across species and is dependent upon specialized phagocytic cells. These cells largely consist of monocytes and macrophages in vertebrates and haemocytes in invertebrates, and play a major role in the recognition and elimination of foreign threats. They are able to mount a defense response against foreign objects by secretion of inflammatory cytokines and chemokines, phagocytosis and, following elimination of the danger, they are also heavily involved in the resolution phase of inflammatory reactions. Furthermore, recent studies have rejuvenated interest in the concept that, following resolution, innate immune cells display non-specific memory of previous stimulation, which alters subsequent innate defense responses. Based upon this concept, known as innate immune memory, we have primed human monocytes in culture with either gold engineered nanoparticles (Au ENP) or LPS. We have undertaken to treat cells in both 2D and 3D conditions, evaluating whether culture conditions affect memory responses. While LPS priming initiated a strong inflammatory response, demonstrated by increased TNFα, IL-8, IL-1Ra and IL-6 production, Au ENP priming did not induce cytokine production in either culture condition. Following a resting period of 5-7 days, cells were again challenged with LPS or Au ENP. No cytokine production was noted in response to Au ENP challenge, while LPS challenge of rested cells resulted in cytokine production that was lower than the initial response of fresh cells. Of the two culture conditions, 3D culture resulted in elevated reactivity compared to 2D, both in the priming and challenge phases. In 2D culture, LPS primed cells that were then challenged with LPS demonstrated reduction of the cytokine response compared to unprimed control cells. Interestingly, in 3D culture this modulation was lost or altered in the cases of IL-1Ra, IL-8, and IL-6, while in TNFα down-regulation was retained. Both the magnitude and the direction (increase or decrease) of the altered memory response appeared to be donor-dependent. Au ENP appeared to have a role in the memory response in 3D culture, a response that is also donor-dependent. These observations demonstrate a role for ENP driven memory in innate immune responses, and that the conditions in which these responses are measured are critical.

Real time PCR and image-based cell profiling analyses evidence correlation between hematopoietic and ampulla cell populations in the gastropod *Pomacea canaliculata*

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Pomacea canaliculata is a freshwater snail gaining visibility among the scientific community due to both its biology and environmental invasiveness. In previous studies, the hematopoietic tissue of this snail has been identified in the pericardial cavity and we hypothesized that the ampulla, a hollow organ directly connected to the heart and located into the pericardial cavity, may play a role in hemocyte maturation. In order to test this hypothesis, different approaches have been adopted.

By real time PCR, we demonstrated that the hematopoietic tissue and the ampulla present similar levels of expression of the prokineticin-like protein, *Pc*-PlP. Conversely, preliminary experiments suggested that the expression levels of a second prokineticin domain-containing protein, *Pc*-Astakine, are significantly different between hematopoietic tissue and ampulla.

These molecular data have been correlated with those collected with ImageStream® X Mark II Imaging Flow Cytometer. This instrument, combining flow cytometry and microscopy technologies, rapidly collects images of thousands of cells and allows high-throughput analysis of cell morphologies and their frequency in different samples. Freshly dissociated or fixed hematopoietic tissue and ampulla have been run. Shared cell morphologies have been observed but the largest part of their frequencies have been shown to be significantly different between the two organs.

Our results suggest that, beyond the apparent differences, hematopoietic tissue and ampulla may share immune-related gene expression and cell populations. The availability of genome sequence and organ-specific transcriptomes for *P. canaliculata* and the recent optimization of *in situ* hybridization protocols, will help to further clarify these observations and to elucidate the role played by the ampulla in hemocyte maturation.

Effects of silver nanoparticles on *Mytilius galloprovincialis* hemocytes and embryos

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Silver nanoparticles (AgNPs) are more and more used in consumer products partly due to its biocidal action. As coastal sessile organisms, mussels are likely to be more impacted by NPs in reason of their filter feeding ability. In this work, the effects of uncoated AgNPs (47 nm) on the marine mussel *Mytilus galloprovincialis* were evaluated at the cellular and whole organism level utilizing both immune cells (hemocytes) and developing embryos. The effects were compared with those of ionic silver (AgNO3). Hemocytes were exposed *in vitro* for 30 min to AgNP suspensions in ASW at several concentrations (1-100 µg/ml). Several functional parameters were evaluated (lysosomal membrane stability-LMS, extracellular ROS production, phagocytosis). The effects of AgNPs on the actin cytoskeletal structures and mitochondrial membrane potential were also investigated using fluorescent dyes by confocal fluorescence microscopy. The embryotoxicity test was performed exposing fertilized eggs to different concentrations (0.001-1000 µg/L) of AgNP suspensions in 96-microwell plates, and the percentage of normal D-larvae was evaluated after 48 h. The results showed that AgNP mainly targeted lysosomes, cytoskeleton and mitochondria of the hemocyte. Toxicity of AgNPs was much lower than that of Ag^+, as indicated by the EC50 values obtained for LMS (273.1 and 1.23 µg/µL, respectively). Regarding the embryos, AgNPs induced a dose-dependent decrease in normal larval development, with concentrations > 80 µg/L inducing developmental arrest. Also in the embryotoxicity test, the effects of AgNPs were lower than those of Ag^+ (EC50 23.71 and 1 µg/L, respectively). Despite the formation of agglomerates in exposure medium, AgNPs were able to negatively interact with both hemocytes and developing embryos. These observations could be bothersome not only for the immune function of mussels but also for future mussel population establishment and potentially hinder their development until the adult stage. These results highlight the fast response of the mussel immune system and strengthen the suitable use of early embryos as sensitive targets for the adverse effects of NPs in marine invertebrates.

Session 2. Chairmen: Loriano Ballarin, University of Padua, Padua, Italy and Davide Malagoli, University of Modena and Reggio Emilia, Modena, Italy
Invertebrate immunity (1)

Myticalins: towards a link between sequence diversity and antimicrobial function

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Myticalins are a recently described family of linear cationic antimicrobial peptides taxonomically restricted to marine mussels (*Mytilus* spp.). While our findings permitted to confirm a relatively broad spectrum of activity towards both Gram-positive and -negative bacterial strains, the functional implications of the puzzling molecular diversity of the AMPs still remain unexplained. Indeed, the "myticalin inventory" shows significant intraspecific variation, both in terms of gene number and presence/absence of particular sequence subfamilies, as confirmed from whole-genome resequencing data. In summary, each mussel is

endowed with a potentially unique set of myticalins. This surprising inter-individual diversity might be linked to benefits at the whole-population level, fitting with the concept of ecological immunity.

The localized expression of myticalins in the gill tissue, a major interface with the external environment, suggests that these AMPs play a role as a first line of defense towards invading microbes acquired from the water column. Unfortunately, classical microbiological assays can only provide preliminary indications about these microbial targets, as the vast majority of marine bacteria are non-culturable. We developed a new method to detect significant shifts in the composition of the gill-associated microbiota following myticalin treatment, based on the metabarcoding of bacterial 16S rRNA.

Other unanswered questions concern the mode of action of myticalins. We demonstrate that, unlike other proline-rich AMPs, myticalins do not exploit the bacterial inner membrane transporter Bac7 to enter target cells. On the other hand, myticalins display marked permeabilizing properties on eukaryotic cells at MIC concentrations. This, together with the complete inhibition of the antimicrobial activity in marine broth, suggests that myticalins act intracellularly on phagocytosed bacteria, without adopting secondary structures, as evidenced by circular dichroism.

Myticalins: a novel multigenic family of linear, cationic antimicrobial peptides from marine mussel (*Mytilus* spp.)

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The application of high-throughput sequencing technologies to non-model organisms has brought new opportunities for the identification of bioactive peptides from genomes and transcriptomes. From this point of view, marine invertebrates represent a potentially rich, yet largely unexplored resource for *de novo* discovery due to their wide range of adaptation strategies to challenging habitats. Thorough bioinformatics analyses of available genomic and transcriptomic data we succeeded to identify myticalins, a novel family of antimicrobial peptides (AMPs) from the mussel *Mytilus galloprovincialis*, and a similar family of AMPs from *Modiolus* spp., named modiocalins. Their coding sequence encompasses two conserved N-terminal (signal peptide) and C-terminal (propeptide) regions and a hypervariable central cationic region corresponding to the mature peptide. Myticalins are taxonomically restricted to Mytiloida and they can be classified into four subfamilies. These AMPs are subject to notable interindividual sequence variability and possibly to presence/absence variation. Functional assays performed on selected members of this family indicate a remarkable tissue-specific expression (in gills) and a broad spectrum of activity against both Gram-positive and Gram-negative bacteria. Overall, we present the first linear AMPs ever described in marine mussels and confirm the great potential of bioinformatics tools for the *de novo* discovery of bioactive peptides in non-model organisms.

A phylogenetic perspective of the cytokine MIF in bivalves

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Macrophage migration inhibitory factor (MIF) is a key regulator of vertebrate immune systems, known to be involved in many other processes, from normal development to diseases. Owing to its multiple roles and its capacity to finely tune several molecular pathways, MIF has been renamed in the 'Most Interesting Factor'. However, the evolutionary history of this cytokine is not limited to vertebrates, given that MIF-like genes have been identified in protostomes, plants and even in bacteria. Notably, MIF-based interactions of some parasites have been reported as a central node to hijack host immune system. Only a few MIF sequences have been identified so far in bivalves, for instance in *Mytilus galloprovincialis*, *Magallana gigas* and *Meretrix meretrix*.

With the aim to update the phylogenetic description of MIF, we searched for *MIF*-like sequences in genomic and transcriptomic data of bivalves, retrieving a total of 148 sequences (80 % of them were previously unknown). We demonstrated that, like vertebrates, most of the analyzed bivalve species encode two *MIF* genes, namely one *MIF* and one *DD-T* gene. Moreover, the genomic expansion of *MIF* genes was evident in two bivalve families, a condition found only in the genomes of obligate blood or lymph-feeding ecto-parasites.

To investigate the role of MIF in marine mussels, we exploited RNA-seq and qPCR data. In *M. galloprovincialis*, expression data only partially support the involvement of *MIF*-like genes in immunity and suggest that MIF and DD-T may be involved in different functional pathways. The ongoing recombinant production of *M. galloprovincialis* MIF in *Pichia pastoris* should provide a valuable tool to clarify its functional role(s) in an invertebrate model of study.

Molecular characterization and expression analysis of the first Porifera tumor necrosis factor superfamily member and of its putative receptor in the marine sponge *Chondrosia reniformis*

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Tumor necrosis factor (TNF) is a pro-inflammatory cytokine involved in a number of cell signaling pathways such as immune function, inflammation, apoptosis, cell differentiation and proliferation. It belongs to a large family of structurally related proteins known as "TNF ligand superfamily" (TNFSF). In the present study, we report the first molecular cloning of a TNFSF member (chTNF) and of its putative receptor (chTNFR) in Porifera, the oldest metazoan group still extant on our planet.

Full-length cDNAs were isolated and cloned from the marine sponge *Chondrosia reniformis*. The deduced chTNF amino acid sequence indicates that this sponge TNF-like is a type II transmembrane protein containing the typical TNFSF domain. Phylogenetic analyses reveal that it is related to abalone disk TNF.

chTNF and chTNFR tissue expression profile was evaluated by qPCR indicating that they are constitutively expressed both in the ectosome and in the choanosome of the sponge, with higher levels in the ectosome. In order to establish the immunological role of the TNF-like protein and to evaluate the presence in sponges of a first ancient TNFR towards which the TNF-like protein may be directed to, the mRNA expression level of the chTNF and of chTNFR were investigated after sponge tissue explants treatment with Gram positive (*Enterococcus faecalis)* or Gram negative (*Vibrio alginolyticus* and *Vibrio fluvialis*) bacteria for 3, 6, 24 and 48 hours. The obtained results indicated that chTNF was significantly upregulated in Gram positive-treated fragmorphs as compared to controls, while chTNFR was upregulated by both treatments. Finally, to investigate the possible involvement of sponge chTNF in collagen biosynthesis, the expression levels of a fibrillar and of a non fibrillar collagen gene were evaluated after *C. reniformis* tissue explants treatment with specific TNF inhibitors and after Gram positive bacterial infection. The obtained results indicate that the cytokine is involved in sponge collagen deposition and homeostasis as described in higher animals.

Allograft inflammatory factor-1 (AIF-1) in the common sea urchin *Paracentrotus lividus*: molecular and expression analysis

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Allograft Inflammatory Factor1 (AIF1), alias ionized calcium-binding adapter molecule 1 (IBA1), is a highly conserved Ca^{2+}-binding cytokine that has been identified as a key regulator of the immune response in vertebrates. AIF1 is highly expressed in activated macrophages during inflammatory responses, thus representing an accurate indicator of macrophage activation in the body and a pathogenic factor in several inflammatory diseases. Proteins of the AIF1 superfamily are also present in invertebrates, from sponges to echinoderms.

Here, we describe the *Paracentrotus lividus Aif-1*, which encodes a predicted protein of 151 amino acids with high similarity to vertebrate AIF1. In the common sea urchin, molecular and immunocytochemical analyses showed the constitutive expression of *Aif-1* in the coelomocytes. Aif-1 localizes in the perinuclear area of amoebocytes and inside the granules of red cells, but it is not present in vibratile cells and colorless spherula cells. Moreover, significant increase of *P. lividus Aif-1* expression, at both mRNA and protein level, are observed in coelomocytes after Gram+ bacterial challenge.

BLAST searches across Echinoderm databases resulted in identification of orthologous proteins from 24 species (8 sea urchins, 1 brittle star, 12 starfishes and 3 sea cucumbers). Among these, *P. lividus* Aif-1 shared a high identity with several species, *e.g.,* 85.4% with the sea urchin *Strongylocentrotus purpuratus*, 60.9% with the brittle star *Ophiocoma echinata*, 59.6% with the starfish *Achantaster planci*, and 52.3% with the sea cucumber *Apostichopus japonicus.*

Our study on *P. lividus Aif-1* will contribute to elucidate AIF1 function along the evolutionary scale and to consolidate the key evolutionary position of echinoderms throughout metazoans with respect to the common immune paths.

TLR2 expression in medicinal leech

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It is well known that Toll-like receptors (TLRs) play a central role for innate immunity in both vertebrates and many invertebrates, by recognizing conserved pathogen-associated molecular patterns in order to trigger the innate immune response. In the case of Gram-positive bacteria, TLR2 is the primary receptor to detect them and mainly senses lipoteichoic acid LTA (a predominant surface glycolipid of Gram+). In order to evaluate whether the recognition of Gram+ bacteria involves TLR2 in leech as well, we performed immunofluorescent and Western blot analysis on leech body wall LTA injected. Our morphological and histochemical results clearly indicate that LTA induces immune cells migrating toward the stimulated area. The expression profile of TLR2 has been also evaluated by Western blot analysis highlighting the presence of an immunoreactive product at about 100 kDa, in according with that found in vertebrates. Moreover, to characterize the migrating immune cells, we further performed immunofluorescent experiments

using antibodies against the AIF-1 (Allograft Inflammatory Factor-1) and RNASET2 (a member of the ribonuclease T2 family) factors. As we previously demonstrated, the expression of these two markers is enhanced after Gram- bacterial lipopolysaccharides (LPS) infection and is mainly located in activated macrophages. Our results clearly show that after LTA injection, numerous AIF-1+/RNASET2+ macrophages migrate towards the infected area and express TLR2.

Given these encouraging preliminary results, indicating that TLR2 may functions as LTA receptor in leeches as well, our further research will be focused to better clarify TLR2 expression and signaling in the medicinal leech.

TLR4 expression in medicinal leech

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Given the enormous vastness and diversity of pathogens, one of the fascinating problems that immunology has to understand is how organisms detect the presence of infectious agents and destroy the invader without damaging the tissues of the organism itself.

In this study, we take into consideration Toll-like receptor 4 (TLR4), which plays a key role in the innate immune response, by using an invertebrate animal model, the medicinal leech (*Hirudo verbana*). One of the advantages of using this model is found in its innate immune system that is very similar to that of vertebrate, but lacking the complex cross-talk typical of adaptive immunity. These features are promising to gain novel insight into the basic mechanisms of the innate immune response.

Here, we evaluate the expression of TLR4 and its co-receptor CD14, after injection in the leech body wall of r*Hm*AIF-1, an endogenous cytokine able to recruit macrophages, or LPS, an exogenous molecule known to induce a strong inflammatory response.

Our results indicate that immune cells migrating toward the body wall of treated animals are macrophages (*Hm*AIF-1+) and granulocytes (CD11b+) and express both TLR4 and CD14. Moreover, the expression profile of TLR4 has been also evaluated by Western blot analysis highlighting the presence of immunoreactive products at about 130 and 110 kDa. We hypothesize that, according to literature, the presence of different molecular masses of TLR4 probably depends on its glycosylation state.

Furthermore, functional studies both *in vivo* and *in vitro* were carried out by using CyP, a cyanobacterium selective TLR4 antagonist, which inhibited the secretion of the pro-inflammatory cytokines, such as tumor necrosis factor alpha. By stimulating with CyP and LPS (Gram- bacterial lipopolysaccharides) or LTA (Gram+ lipoteichoic acid), we found that only TLR4 pathway was blocked, while the immune response caused by LTA treatment was not affected. Our results are consistent with literature on vertebrates indicating that TLR4 functions as LPS receptor while the recognition of Gram+ bacterial products involves other pathways, such as TLR2. Further research is needed to better clarify TLR4 expression and signalling in the medicinal leech.

AIF-1 and RNASET2 play different roles in the modulation of leech innate immune response

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In recent years, several studies have demonstrated that the AIF-1 (Allograft Inflammatory Factor-1) and RNASET2 (a member of the ribonuclease T2 family) factors are involved in the activation and modulation of inflammation processes, both in vertebrates and in invertebrates. In particular, it has been demonstrated that both AIF-1 and RNASET2 have a chemotactic activity for macrophages and that their expression significantly increases after bacterial infections. However, the details on the mechanisms by which these evolutionarily conserved proteins regulate the innate immune response are still poorly defined. In order to better understand the specific role of these two factors, we report here the effect of Gram- bacterial lipopolysaccharides (LPS) injection on AIF-1 and RNASET2 expression in an invertebrate model, the medicinal leech. This animal has been chosen for its very simple anatomy and for its notable similarities during inflammatory processes with those of vertebrates. Western blot assays demonstrate that AIF-1 and RNASET2 have a different temporal expression profile. In fact, while RNASET2 has two expression peaks after 30 min and 6 h from LPS injection, AIF-1 shows a peak after 30 min and its expression remains high after 24 h from stimulation. Furthermore, double immunostaining coupling anti-AIF-1 or anti-RNASET2 to an antibody directed against the common granulocyte marker CD11b demonstrates that these two factors are expressed by two different types of cells, whose amount varies over time after LPS stimulation. RNASET2 is mainly localized in the granulocytes, the first cells migrating towards the stimulated area, AIF-1 is instead expressed by macrophages.

Taken together, our results clearly suggest that AIF-1 and RNASET2 play a different role in the initial phase of the inflammatory response. We suggest that RNASET2 is at first released by granulocytes in order to induce a massive recruitment of macrophages. Once chemoattracted to the stimulated area, activated macrophages highly express AIF-1 to sustain the recruitment of further macrophages whose role is to rid the area of bacteria.

Preliminary data on a Toll-like receptor from the colonial ascidian *Botryllus schlosseri*

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Toll-like receptors (TLRs) represent a well-known family of conserved pattern recognition receptors the importance of which, in non-self recognition, was demonstrated in both vertebrates and invertebrates.

Tunicates represent the vertebrate sister group and, as invertebrates, they rely only on innate immunity for their defense. As regards TLRs, two transcripts have been described and characterized in the solitary species *Ciona intestinalis*, referred to as CiTLR1 and CiTLR2. Using the *Ciona* TLR nucleotide sequences, we examined the genome and the available transcriptomes of *Botryllus schlosseri* looking for similar sequences. We were able to identify a sequence, with similarity to CiTLR2 and, through *in silico* transduction and subsequent sequence analysis, we studied the domain content of the putative protein. The sequence, called BsTLR, has a TIR and a transmembrane domain, four LLR and two LRR-CT domains. In addition, we analized *Bstlr* expression *in vivo* and *in vitro*, under various experimental conditions and in different phases of the *Botryllus* blastogenetic cycle. Our data show that, in different phases, there is a change in gene expression and mRNA location, according to the blastogenetic phase.

Session 2. Chairmen: Magda de Eguileor, University of Insubria, Varese, Italy and Paola Venier, University of Padua, Padua, Italy
Invertebrate immunity (2)

The case of Ostreid herpesvirus 1 (OsHV-1) and the antiviral host's countermeasures

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Prevention and mitigation of infective bivalve diseases is the central objective of the current European project VIVALDI (2016-2020, Research and Development programme H2020) coordinated by the Institut français de recherche pour l'exploitation de la mer (IFREMER, I. Arzul). Here, we present an overview on a current problem and the results achieved so far with the support of European grants (BIVALIFE, VIVALDI).

Herpesviruses infecting marine molluscs have been associated to recurrent mortality outbreaks worldwide and, still today, cause serious production losses. Described in France since 1991-1993, the acute and anomalous mortality occurring in hatchery larvae as well as in spat and juveniles of Pacific oyster *Magallana gigas* is usually detected at the increasing temperatures of Spring-Summer transition in association with the infection with a herpes-like virus. Possibly triggered by multiple causes, the oyster mortality rates can reach 80-100% in less than a week. Hence, this is an issue somewhat different from the summer mortality of adult oysters more often interpreted as a physiological disorder related to overgrowth and gonad over-maturation.

Electronic transmission microscope (TEM) observation of herpesvirus-like particles in gill and mantle tissues of muribund oysters and positive PCR-based diagnosis pointed to a viral etiology already in the early '90s in France. A herpesvirus purified from naturally infected *M. gigas* larvae originated the first genome sequence (a dsDNA of about 207 kbp, GenBank accession number AY509253) and permitted the classification of Ostreid herpesvirus type 1 (OsHV-1) within the Malacoherpesviridae family from the Herpesvirales order (Davison *et al*., 2009). More virulent OsHV-1, called microvariants because of a microdeletion and other nucleotide changes, emerged in 2008-2010 in France, Europe and subsequently could be diagnosed in farmed *M. gigas* worldwide. In addition to the precise knowledge on diagnostic sequence regions, two microvariant OsHV-1 genomes from French and Irish oysters are now available and more ones will likely appear. Molecular data resulting from natural and experimental OsHV-1 infections and from the transcriptome analysis of OsHV-1-infected host will be discussed as knowledge basis for prevention and mitigation strategies.

Molecular basis of melanization in bivalves

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Melanization is part of the ancestral defense systems of invertebrate phyla such as arthropods and mollusks and commonly participate to wound healing and encapsulation of invading microbes or parasites. Melanin synthesis and deposition has been associated to pigmentation, exoskeleton production or biomineralization and it is relevant during larval development. The chemical reactions leading to melanization are based on the sequential activity of phenoloxidases (PO such as tyrosinases, laccases or catecholases) and dopachrome converting enzymes (DCE or DCT).

Here, we review the candidate molecules possibly involved in the melanization process in bivalves. Recent studies indicate a lineage-specific expansion of bivalve tyrosinase-like genes. Their gene expression patterns, together with the neofunctionalization of some paralogs, support the dual role of tyrosinases in immune defense and shell biomineralization. Tyrosinase enzymes might enable the biosynthesis of melanin starting from the precursors dihydroxyindole (DHI) or dihydroxyindole-2-carboxylic acid (DHICA). However, the nature of the enzymes involved in the generation of these molecules from L-Dopachrome is presently unknown in bivalves, due to the absence of both DCT and DCE-like sequences.

We report the expansion of D-Dopachrome Tautomerase genes in bivalves. Moreover, their mantle-specificity and inducibility upon bacterial challenge, together with their enzymatic activity suggest an involvement in melanin biosynthesis. However, the activity of these enzymes to promote the conversion of L-Dopachrome to DHI or DHICA in the melanin pathway still remains to be established.

Survival and immunological competence of *Procambarus clarkii* after X-ray irradiation

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The Louisiana's red swamp crayfish (*Procambarus clarkii*), native to the Southern United States and currently present all over the world, competes with native species and is a vector of crayfish plague caused by the water mold *Aphanomyces astaci*. *P. clarkii* is also reported among the 100 worst invasive species (DASIE) and among the 9 ones with the worst impact on more than 4 threatened species (Genovesi *et al*., 2015).

The Sterile Male Release Technique (SMRT) represents one of the newest controlling methods for invasive crustaceans and in combination with intensive trapping has been proved to be particularly effective in the lake of Casette (Pordenone, Friuli Venezia Giulia, Italy) with a reduction of about 87% of *P. clarkii* population after two years of activity (Aquiloni and Zanetti, 2014). In a previous study, short-term effects of x-ray have been obtained and analysed after 20 days from the irradiation of *P. clarkii* males (Giglio *et al*., 2018). The present study investigates the long-term effects of X-ray irradiation of a dose of 40 Gy on 19 males after about 6 months from the exposure. Immune competence has been evaluated by analyzing total hemocyte counts (THC), and activity of basal and total plasmatic prophenoloxidase. Glycemia, as a generic stress index, and total plasmatic proteins were also measured. Hemolymph withdrawals were performed at 5, 12, 28, 35, 65, 99, 132 and 193 days post treatment. Testicular damages, at the end of experiment, were assessed by means of measurements of acini in semithin sections of fixed testis. The mean death days after treatment were 44.21 ± 4.15 for irradiated animals and 82.71 ± 19.47 for controls. The survival model with constant hazard estimates an average death age for irradiated animals of 113.14 days and for controls of 165.43. Irradiated and control groups do not differ in survival rate ($p = 0.33$) and activity of plasmatic prophenoloxidase ($p > 0.12$). The THCs of irradiated animals are significantly lower than those of untreated animals 5, 12, 28, 65, and 99 days after treatment ($p < 0.05$). Basal plasmatic PO activities of irradiated animals are significantly lower at 132 and 196 days after irradiation. Our findings document that despite a significant damage to the immune cell component, the humoral prophenoloxidase activity do not differ between irradiated and control animals. Surprisingly, whilst after 1 month from irradiation an extensive gonadal damage was described (Piazza *et al*., 2015), after 6 months in surviving crayfish no significant differences in acini diameters were recorded. Our data confirm the validity of SMRT for managing *P. clarkii* in confined basins with the indication of radiating large males a few days before the reproductive season considering life expectancy of about 80 days after treatment. Furthermore, our data demonstrate that cytological damage in gonadal tissues could be repaired in 6 months after irradiation with a functional recovery.

Amyloid and immune responses in the colonial ascidian *Botryllus schlosseri*

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Increasing evidences indicate that functional, non-toxic amyloid is widely distributed among living organisms. In invertebrates and vertebrates, functional amyloid is involved in inflammatory reactions and modulation of immune responses. In the present study, we investigated the occurrence of functional amyloid in tunicates, the closest living relatives of vertebrates. Previous studies indicate that orthologous genes for the amyloid precursor protein (APP), involved in amyloid synthesis in vertebrates, are present in solitary ascidians of the genus *Ciona*. In addition, colonies of compound ascidians of the genus *Botryllus*, respond to contacting genetically incompatible colonies with an allorecognition reaction that leads to the formation of necrotic, melanic spots along the contact border. Our data provide evidence that functional amyloid is involved in immune responses of *Botryllus schlosseri* and indicate that, both the circulating immunocyte types, *i.e*., the cytotoxic morula cells and phagocytes, can produce amyloid using two different proteins: Bsp102 and BsAPP. Bsp102 forms the amyloid functioning as scaffold to store MC granular content and, once released upon MC degranulation, the support where phenoloxidase and melanin are deposited, thus limiting the diffusion of cytotoxicity. BsAPP is released by phagocytes and contribute to the formation of extracellular nets that entrap microbes and prevent their diffusion within the organism. To the best of our knowledge, this is the first report of functional amyloidogenesis in protochordate immunity.

Purification of Galactose binding lectin from the mucus of *Sabella spallanzanii* (Gmelin, 1791) and interaction of arsenic with bacterial agglutinating activity

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Lectins are present in almost all living organisms and are involved in several biological processes, including immune responses. In the present study, a galactose binding lectin (SsGBL) exhibiting an apparent MW of 43 kDa has been characterized and purified from the mucus of the polichaete *Sabella spallanzanii* by using both affinity chromatography and high-pressure liquid chromatographic methods. Its agglutinating activity towards rabbit erythrocytes was significantly modified by the addition of calcium or EDTA. The activity was optimal at temperature values comprised between 4 °C and 37 °C, was partially retained after exposure at 50 °C, and was depleted at 90 °C. The SsGBL was able to agglutinate bacteria. The strongest activity was observed towards *V. alginolyticus* and *E. coli*, by contrast SsGBL at lesser extent agglutinated the Gram positive *Micrococcus lysodeikticus*, suggesting its possible involvement in host pathogen interactions.

Chemical analysis of animal tissues shows high concentrations of arsenic, in the branchial crown of animals (Bio Accumulation Factor: 1,869) respect to sea water. We then investigated the arsenic effect and according to the branchial crown accumulation the mucus bacterial agglutinating activity results show that the presence of arsenic determines a modification but not complete inhibition also at the higher used concentration (BAF ranging from 200 to 20,000), whereas methylmercury totally depletes the mucus agglutinant activity.

Mytilus galloprovincialis hemocytes activity as potential biomarker of marine pollution: *in vitro* effects of organic mercury

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Hemocytes found in hemolymph, constitute a heterogeneous population of immune cells involved in defences against pathogens including non-self recognition, encapsulation and generation of cytotoxic molecules. These immunocompetent cells are also functionally and morphologically responsive to various xenobiotics present in the aquatic environment.

Toxic metals, such as mercury, contribute substantially to anthropogenic pollution in many coastal environments. Animals living in those environments, particularly invertebrate filter feeders like bivalves, can be used as bioindicators. In an attempt to identify cellular markers for revealing pollution, this study examined *in vitro* the effects of different concentrations of methyl mercury on *Mytilus galloprovincialis* hemocytes activities and morphology. As immunological parameter of cell-mediated immune response, hemocytes mortality and morphology change, within the permissible physiological limit, were evaluated adding three concentrations of MeHg in MS to the cultured cells. To evaluate the physiological status of the organisms, responses at the cellular and molecular biological organization were measured through *in vivo* cytochemical method.

The Neutral Red uptake in lysosomes test was performed on withdrawn hemocytes and cytotoxic activity was evaluated toward various target by spectrophotometric measurement of the released hemoglobin and Plaque-forming cell assay. In particular, hemocytes exposed to the xenobiotic presented a significantly decrease in the phagocytic activity toward yeast. The cytotoxic activity of *M. galloprovincialis* hemocytes toward erythrocytes and plaque lysis activity was not altered by suitable methylmercury concentrations in the medium. In both the responses cell-target contacts could be affected by methylmercury. The harvested hemocytes that were exposed to the metal had a significant mortality, cellular count and morphometric alterations. These findings provided evidence of MeHg immunotoxic effects resulting in hemocyte death and morphological changes induced by cytoskeleton alterations. Thus, a morphometric cellular parameter, such as spreading ability, was used as a complementary method and results confirm that MeHg is toxic for *M. galloprovincialis* hemocytes, causing immunosuppression either by cell death or morphological changes.

Involvement of the Multixenobiotic Resistance (MXR) system in the physiological chemoresistance of haemocytes in marine mussels

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In bivalves, the immune response is mainly supported by haemocyte cells found in the open hemolymphatic circulatory system. Haemocytes are responsible for cell-mediated immunity through phagocytosis and different cytotoxic reactions, and are targets for the effects of several environmental pollutants. Therefore, haemocytes are biological models to explore pollutant effects on bivalve immunity, and to infer the role of protective mechanisms triggered in these cells under immunotoxicological reactions.

This study investigated functional and transcriptional modulation of the Multixenobiotic resistance (MXR) system as a cytoprotective mechanism contributing to the physiological chemoresistance of haemocytes in the mussel *Mytilus galloprovincialis*. The MXR system allows aquatic organisms to cope with their habitat despite high pollution levels. ATP-binding cassette (ABC) transport proteins are integral part of the MXR system, as they perform a crucial step in detoxification processes, *i.e.*, the active efflux of metabolites and xenobiotics over the cell membrane, which results in lower intracellular concentrations and lower toxic potential.

Basal transport activity was assessed using the model substrate rhodamine 123 and specific

inhibitors for the MXR-related transporters P-glycoprotein (*ABCB* mRNA) and Multidrug resistance-related protein (*ABCC* mRNA). Results showed that MXR activity in mussel haemocytes was mainly supported by the Mrp-mediated efflux. In agreement, *ABCC* was expressed at higher levels than *ABCB*. Activation of the cyclic-AMP (cAMP) dependent protein kinase A (PKA) resulted in increased rhodamine efflux, which was counteracted by the selective PKA inhibitor H89. *In vitro* experiments treating haemocytes with physiological agonists (noradrenaline and serotonin) and pharmacological modulators (PROP, forskolin, dbcAMP, and H89) of the cAMP/PKA system showed that serotonin (5-HT) acts as a physiological modulator of *ABCB* transcription in haemocytes. Although under these experimental conditions overall MXR activity was not affected, the environmental pharmaceuticals fluoxetine, propranolol, and carbamazepine, which interact in different ways with the adrenergic and serotoninergic pathways, acted as modulators and substrates of MXR-related transporters and affected cell viability, highlighting the potential of these pharmaceuticals to induce immunotoxicological effects as part of their adverse outcomes in marine mussels.

In vitro effects of different _Ganoderma lucidum_ extracts on _Mytilus_ hemocytes

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Aquaculture is one of the fastest growing food-producing sectors worldwide. However, during intensive farming practices, a major problem is represented by development of various infectious diseases. In this regard, the use of medicinal plant and mushroom extracts that can act as immunomodulators has been proposed to control to prevent and control fish and shellfish bacterial, viral, and parasitic infections.

Many species from the genus *Ganoderma* P. Karst (Basidiomycotina, Polyporales, Ganodermataceae) include a group of fungi that have been widely used in traditional medicine in Asian countries. In particular, the most studied species is *G. lucidum*, that has beneficial health properties due to a wide variety of bioactive components, such as polysaccharides, phenolic compounds, triterpenes, sterols, lectins and some proteins, with different biological activities.

In this work, preliminary data are presented on the possible effects of ethanolic and aqueous extracts of *G. lucidum* on the hemocytes of the marine bivalve *Mytilus*, an important aquacultured species worldwide.

The aqueous extract, containing polysaccharides, induced increases in lysosomal membrane stability, phagocytic activity and oxidative burst, indicating immunostimulatory effects. Dose-dependent effects were observed in the µg/ml range.

In contrast, the ethanolic extract, containing polyphenolic compounds, induced lysosomal membrane destabilization, inhibition of phagocytosis, and oxidative stress.

The results represent the first data on the effects of medicinal mushroom extracts on immune parameters of aquacultured bivalve species. Studies are in progress to evaluate the immunostimulatory effects of *Ganoderma* extracts and fractions in mussels.

Session 3. Chairmen: Luigi Abelli University of Ferrara, Ferrara, Italy and Maria Rosaria Coscia, Institute of Protein Biochemistry, CNR, Naples, Italy
Fish immunity

Fish cytokines: molecular characterization and biological activity of IL-2 and IL-2L in sea bass (*Dicentrarchus labrax* L.).

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Interleukin-2 (IL-2) is a fundamental immunomodulatory cytokine, secreted mainly by T-helper 1 cells, involved primarily in the proliferation, activation and differentiation of T cells. In Teleost fish, the first evidence for the existence of an IL-2 gene was reported about ten years ago and only in rainbow trout (*Oncorhynchus mykiss*) the IL-2 bioactivity has been studied quite in detail. In our work, we have found in a sea bass (*Dicentrarchus labrax* L.) gills transcriptome two sequences related to fish IL-2, a proper IL-2 gene and a IL-2L (IL-2 like) transcript. These two transcripts that have been mapped on the available sea bass genome and the sequences have been confirmed by cloning from a sea bass gills cDNA.

Basal expression analysis by real-time PCR revealed that IL-2 was mainly expressed in gut, with lower expression in brain and spleen, whereas IL-2L in gut. Moreover, we investigated the expression of the IL2 and IL-2L in sea bass head kidney leukocytes after *in vitro* stimulation with the T cell mitogen agent PHA and after *in vivo* infections with nodavirus and *Vibrio anguillarum*. Finally, we produced sea bass IL-2 and IL-2L as recombinant proteins in *E. coli* and we tested their action *in vitro* on the modulation of different immune-related genes expression after stimulation of leukocytes from head kidney. This is a first preliminary analysis of the biological activity of the IL-2 cytokine in sea bass and it will highly contribute to a broader understanding of the evolution of T-cell immunity in this species.

First evidence of T cell restricted intracellular antigen (TIA) protein gene expression in Antarctic fish

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Stress granule (SG) formation is a primary mechanism through which gene expression is rapidly modulated when the eukaryotic cell undergoes cellular stresses (including heat, oxidative, viral infection, starvation). The importance of SGs is seen in several disease states in which SG function is disrupted. Fundamental to SG formation are the T cell restricted intracellular antigen (TIA) proteins (TIA-1 and TIA-1 related protein (TIAR)), that both directly bind to target RNA and self-associate to seed the formation of SGs. The only TIA proteins of Antarctic fish so far present in the NCBI database (three variants of TIA-1 and one TIAR) were those of the black rockcod *Notothenia coriiceps*. With the aim to increase the knowledge on these proteins in Antarctic teleosts, we have characterized the genes codifying for TIA-1 protein variant 2 (TIA-1b) in the emerald rockcod *Trematomus bernacchii* and in the crocodile icefish *Chionodraco hamatus*. In the transcriptome of *T. bernacchii* we have verified the presence of TIA-1b. For *C. hamatus*, a partial cDNA sequence of this gene was obtained by RT-PCR technique, with primers designed after cross analyses between NCBI and *T. bernacchii* transcriptome databases. Multi-alignment analysis, performed with fish orthologous sequences, demonstrated high conservation of amino acids. The gene transcription of TIA-1 from various tissues (gills, heart, liver, spleen, and skeletal muscle) of both *T. bernacchii* and *C. hamatus* has been measured by quantitative Real Time PCR. The tissue-specific differences in the TIA-1 mRNA accumulation are probably related to the physiological function characteristic of these organs (Supported by P.N.R.A. and M.I.U.R. grants).

Evolutionary analysis of Immunoglobulin T genes from Antarctic fish

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In 2015, our group isolated *IgT* heavy chain constant region gene from the Antarctic teleost *Trematomus bernacchii* (family Notothenidae), disclosing a unique feature: the loss of most heavy chain second constant domain (CH2). *T. bernacchii* belongs to the Perciform suborder Notothenioidei which represents the main component of Antarctic fish fauna. This teleost group has acquired peculiar features to adapt to the extremely cold Antarctic environment, providing an extraordinary model system for investigating the relationship between evolutionary genomic changes and environmental adaptation. Notothenioidei comprise five Antarctic families, Channichthydae, Bathydraconidae, Artedidraconidae, Nototheniidae, and Harpagiferidae, and two non-Antarctic families, Eleginopidae and Bovichtidae. In an attempt to reconstruct the loss of CH2 through phylogeny of Notothenioids we isolated and characterized *IgT* genes from two more species belonging to family Nototheniidae, one representative of two other Antarctic fish families, (family Bathydraconidae and family Artedidraconidae). Moreover, one representative each of the two non-Antarctic families was included in our studies for comparison: *Eleginops maclovinus* (family Eleginopidae), and *Bovichtus diacanthus*, (family Bovichtidae). The former was proposed as the closest sister group to all the rest of Notothenioids, the latter was chosen for comparison since it represents the phyletically basal lineage of Notothenioid species that inhabited non-Antarctic more temperate waters before Antarctica became isolated from other continents. Genomic studies have been carried out in an attempt to define some events, such as insertion of transposable elements, alternative splicing or other molecular mechanisms, involved in leading to the lack of the most CH2 domain. A comparative analysis at genomic level has highlighted that the presence of a remnant CH2 domain is shared to all Antarctic fish families analyzed in the present work. It is worth of note that the loss of most CH2 is shared also by *E. maclovinus*. This result may be viewed as a preadaptation sign accompanying the lineage radiation.

Session 4. Chairmen: Pierangelo Luporini, University of Camerino, Camerino, Italy and Piero Giulianini, University of Trieste, Trieste, Italy
Recognition and immunomodulation

Insights into the molecular basis of self/non-self recognition in the ciliate *Euplotes* from the determination of pheromone crystal structures

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Species-specific families of protein pheromones, each encoded by one of a series of co-dominant alleles at the same genetic locus (the mat locus), are constitutively secreted by species of *Euplotes* and used as autocrine (autologous, or self) signals that promote the cell vegetative growth and paracrine (heterologous, or non-self) inducers of cell-cell unions in mating pairs. In *E. raikovi*, the structures of a number of pheromones (designated E*r*-1, E*r*-2, E*r*-3 and so forth) have been determined by solution NMR spectroscopy, and the structure of pheromone E*r*-1 has additionally been solved also by X-ray crystallography. In each cell type, the soluble pheromone finds a structural counterpart

with the extracellular ligand binding domain of its membrane receptor, both the molecules being encoded by the same gene through a mechanism of intron-splicing. Given this genetic context, the protein-protein interactions in the crystals can be assumed to mimic those underlying the pheromone/receptor interactions on the cell surface. We used non-conventional *ab initio* crystal structure determination methods, that exploit the high-resolution of collected diffraction data, to compare the crystal structures of two pheromones, E*r*-1 (structure re-determined at a resolution of 0.7 Å) and E*r*-13 (structure *de novo* determined at a resolution of 1.4 Å), that are secreted by two strongly mating compatible cell types. In spite of sharing the same disulphide bond pattern and the same up-down-up three-helix fold, E*r*-1 and E*r*-13 differ markedly in the arrangement of the molecules in the crystals. The resulting intermolecular contacts assign E*r*-1 and E*r*-13 to distinct crystallographic space groups (C2 and P41, respectively), suggesting that the autocrine pheromone/receptor interactions on the cell surface are likely regulated by the capability of each pheromone to homo-oligomerize with an its own specific pattern. In addition, although adopting distinct crystal structures, E*r*-1 and E*r*-13 make a common use of their helix 3 to stabilize the inter-molecular crystal contacts, which implies that this helix likely plays a central role in allowing cells to establish paracrine pheromone/receptor interactions.

Silica-induced fibrosis: from a physiological response in the early metazoans to the well-known pathological outcomes of higher vertebrates

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Exposure to fine crystalline silica particles (quartz) causes silicosis, an occupational disease leading to an overproduction of collagen in the lung. On the other hand, in the invertebrate world, the marine sponge *Chondrosia reniformis* is able to incorporate and partially dissolve crystalline silica grains without toxic effects by means of high concentrations of ascorbic acid (AA) able to erode the crystalline silica surface. In fact, quartz erosion in *C. reniformis* seems to stimulate a physiological process of collagen deposition probably due to the necessity to strengthen the body structure since the animal does not produce spicules by itself. This primitive process is specific for the crystalline silica, the amorphous one, even if equally incorporated by the sponge, remain untouched. This could be the cause of the subsequent abnormal, pathological response to quartz in higher Metazoa, namely Mammals, which retain the ability to interact and engulf the quartz particles through macrophage activation but have lost the ability to dissolve them. This unresolved process leads to a chronic inflammation which finally causes the development of silicosis. Our studies point out as, in mammalian macrophages, AA-pretreated quartz, mimicking the sponge erosion process on the crystalline particles, acquires a high cytotoxic as well as pro-inflammatory potential leading to a significant increase of inflammatory mediators such as TNF, COX2 and PGE2 production as well as ROS and lipid peroxide generation. These effects are due to the abundance of oxygen radicals generated on the crystalline silica particle surface after ascorbic acid interaction. Also, primary human fibroblasts show an abnormal response to AA-pretreated quartz particles. In fact, in fibroblasts, the abundance of surface radicals on quartz crystals significantly enhances cell proliferation, ROS production, NF-kB nuclear translocation, smooth muscle actin, fibronectin, Bcl-2 expression and collagen production. These *in vitro* results may be relevant to the comprehension of the *in vivo* onset of silica-induced pathologies in the lung since ascorbic acid, as in *C. reniformis* marine sponge, is present in significant amounts in the lung epithelium surfactant and could indeed participate to the chronicization of quartz toxicity in this organ renewing the particle surface radicals *ad libitum* and thus perpetrating the inflammatory response which ultimately leads to the development of the silicotic disease.

Immunoregulatory and pro-tumoral effect of circulating DNA

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Circulating DNA (cDNA) is detectable at low concentration in the plasma of healthy subjects and at high concentrations in the peripheral blood of pregnant women and patients affected by tumors and vasculitis. DNA is considered a danger signal mediating pro-inflammatory reactions after binding to DNA sensors. However, this view conflicts with recent data on DNA-sensors function and with the presence of elevated cDNA concentrations in tumor patients, where processes related to immune tolerance generally prevail, leading to variable degrees of immunodeficiency. Hence, the biologic role of cDNA is still controversial. Previous data from our group suggested that DNA may exert *in vitro* immunoregulatory functions interacting with HLA class II molecules. Here, we show that exogenously administered cDNA mediates immunoregulatory functions *in vivo*. In particular, it protects lupus-prone mice from disease progression and favors tumor growth in tumor-challenged mice. Interestingly, cDNA was found associated only to cells expressing MHC class II molecules and its binding to mouse MHC class II molecules was demonstrated. Some of the mechanisms underlying cDNA immunoregulatory functions were elucidated and consisted in regulatory T cell (Treg) expansion,

increased monocyte production of CCL22 (a chemotactic factor for Treg) and inhibition of antigen-specific T cell proliferation. Hence, this study unveils unprecedented biologic functions of cDNA that may have pathogenic relevance in cancer.

Impact of marine contaminants of emerging concern on the cetacean transcriptome

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Contaminants of emerging concern (CECs) are widely distributed in the environment, but their occurrence and potential toxicity are only now being evaluated. CECs are increasingly being detected in the waters and many act as endocrine disruptor compounds (EDCs), causing a variety of effects on health. The worldwide distributed perfluorooctanoic acid (PFOA) and bisphenol A (BPA) are CECs falling in the EDCs category.

Skin samples from the bottlenose dolphin (*Tursiops truncatus*), a top predator that spends its entire life in the water and therefore subject to accumulation and magnification of contaminants, were collected to analyze the impact that environmentally relevant concentrations of CECs may have on global gene expression. We combined transcriptomic analysis and *ex vivo* assays using small skin slices cultured and treated for 24 h with PFOA or BPA or vehicle.

RNA from dolphin biopsies was labeled and hybridized to a species-specific oligomicroarray. The skin transcriptome displayed changes related to contaminant exposure, potentially predictive about long-term effects on health, being the genes affected involved in immune modulation, response to stress, lipid homeostasis, and development. Within the genes differentially expressed in the transcriptome after CECs treatment, 4 were tested as potential gene markers of anthropogenic contaminants exposure on skin samples from wild cetaceans. RNA from 12 individuals, including the species *Stenella coeruleoalba*, *T. truncatus*, and *Grampus griseus* were sampled in 3 areas (Adriatic, Ionian and Tyrrhenian seas). Three out of the 4 genes tested showed higher expression in the samples collected from the Adriatic sea.

The transcriptomic signature of a dolphin skin could be relevant as classifier for a specific contaminant whilst giving information of the specific geographic location where the marine mammal spent its life, due to the different impact on gene expression exerted by different contamination levels.

Amphibian peptides for skin protection and healing

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BACKGROUND: Amphibians are currently suffering a dramatic decline worldwide, mainly due to chytridiomycosis, a skin infection caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd). An important natural defense of amphibian skin is the production of antimicrobial peptides (AMPs) by granular glands in the dermis. AMPs collected from several species of frogs successfully inhibit the growth of Bd *in vitro*. Besides their anti-microbial and anti-fungal activities, AMPs have been shown to exert other biological effects such as anti-viral, anti-tumor, anti-oxidant, immunomodulating and wound healing.

AIM: We intended to test the efficacy of AMPs as cutaneous defenses in frog species either resistant or susceptible to Bd.

METHODS: 3 frog species, *Gastrotheca nebulanastes* (GN), *G. excubitor* (GE) and *Hypsiboas gladiator* (HG), were collected in montane scrub, cloud forest and high elevation grassland habitats near Manu National Park in southeastern Peru. AMP secretion was stimulated by injection of norepinephrine into the dorsal lymph sacks. AMPs were then purified by chromatographic techniques. The human endothelial cell line HECV was treated with AMP concentrations ranging from 0.005 to 50 µg/mL. Cell viability was verified by MTT test. Wound healing properties were analyzed by scratch wound assay. AMP inhibition strength against Bd growth was measured *in vitro* by incubating Bd zoospores with different concentrations of AMPs.

RESULTS: Treatment with AMPs secreted from GN, GE and HG did not affect HECV cell viability at any concentration tested. No significant differences in cell migration rate were observed in HECV cells scratched and treated with GN and GE AMPs. Only HG peptides showed wound healing properties as well as strong Bd growth inhibiting ability.

CONCLUSIONS: Stimulation of wound healing mechanisms and inhibition of Bd growth by skin AMPs might both contribute to HG resistance to chytridiomycosis. Understanding the role of skin defenses may lead to the development of novel Bd mitigation strategies. Possible applications of amphibian AMPs in skin medicine deserve attention and further studies.

Characterization of CD3ε+ T lymphocytes of sea bass *Dicentrarchus labrax L*.

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CD3 is an important cell surface marker of T

lymphocytes and it is essential for T cells activation in higher vertebrates. Here, we report expression analysis and quantitative distribution of the CD3ε+ T lymphocytes in sea bass *Dicentrarchus labrax*. From a whole sea bass gills transcriptome, we identified and successively cloned a complete cDNA sequence of CD3ε. The basal expression of sea bass CD3ε chain in different organs has been analysed by real-time PCR. *In vitro* stimulation with the T-cell mitogen PHA, resulted in a significant increase of the CD3ε expression level compared to control cultures. The CD3ε transmembrane and cytoplasmic tail region has been identified and used to select three peptides for the immunisation of rabbits in order to obtain antisera against CD3ε (Ra CD3ε1). Flow cytometry, IHC and IIF were conducted to address the normal distribution and number of CD3ε+ lymphocyte population in the lymphoid organs (thymus, head kidney and spleen) and mucosal tissues (intestine and gill), with relatively high percentages of these cells identified in thymus, head kidney and spleen, while lower in gills and intestine. At the microscope, the IIF-positive cells had the morphology of lymphocytes and the presence of uniquely stained CD3ε+ IgM-subset cells fits the expected profile of T-cells. The increase of CD3ε expression level found in head kidney leukocytes in response to *Vibrio anguillarum* vaccination suggested that CD3ε+ T lymphocytes might play important roles in the protection against bacterial infections, as in mammals.

Seasonal changes in immune parameters in *Mytilus galloprovincialis* farmed at different sites of the Gulf of La Spezia, Ligurian sea, and their relationship with other biomarkers of the health status

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Marine mussels (*Mytilus* spp.) are worldwide utilized in marine biomonitoring by a multi-biomarker approach. However, for a correct interpretation of different biomarker responses, information is needed on their natural seasonal variability due to environmental stressors/physiological factors. This particularly applies to immune-related biomarkers, that are not currently measured in biomonitoring programs.

The gulf of La Spezia (Ligurian Sea, Italy) is an intensive rearing area for *Mytilus galloprovincialis*. Seasonal variability of 7 different biomarkers, from subcellular to whole organism level, including immune biomarkers, has been recently investigated over 1 year (2015-2016) on a monthly basis in mussels grown at 3 different sites of the aquaculture plant of La Spezia in comparison with a reference site. The data represent the first baseline information on the health status of mussels in this farming area (Balbi *et al*., 2017).

In this work, we report data collected within a project of the Italian Ministry of Health, carried out in collaboration with the IZS of La Spezia*. Mussels were sampled every 3 months (from Nov 2016 to Jul 2017) from the same 4 sites. A set of immune, oxidative stress and inflammation related parameters were evaluated: lysosomal membrane stability-LMS and phagocytic activity of circulating hemocytes, nitric oxide production in the gills as a marker of tissue inflammation, antioxidant enzyme activities (GST, catalase) in both gills and digestive gland. The results were related to determination of mussel survival in air (stress on stress-SoS response) as a biomarker of general health condition at the whole organism level.

The results confirm the seasonal trend previously observed in different biomarkers in mussels sampled at different sites. High immunocompetence was generally observed throughout the year. Differences observed among sites in inflammatory and oxidative stress parameters were mainly due to differences in the gametogenic cycle. The results underline the importance of LMS and SoS as core descriptors of the mussel health status in relation to seasonal variations in temperature and reproduction.