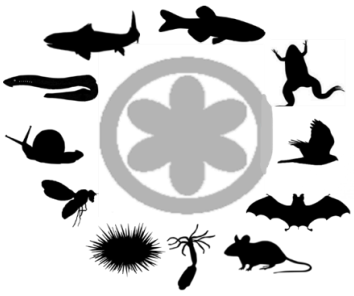

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Abstract Book

Evolutionary Origins and Expression of Antiviral TRIM Proteins in the Duck

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Tripartite motif (TRIM) proteins are involved in antiviral immune responses. The TRIM gene repertoire has not been characterized in ducks, an important ecological reservoir for the spread of influenza A viruses. Here we identify the TRIM gene repertoire in the domestic mallard duck (*Anas platyrhynchos*) by mining publicly available data on NCBI. We show ducks have 57 TRIM proteins which, like human TRIM proteins, are divided into 12 subfamilies based on their C-terminal domains. Among these, TRIM proteins belonging to subfamily C-IV with a C-terminal PRY-SPRY domain are often associated with immune responses in mammals. We inferred phylogenetic relationships between the PRY-SPRY TRIM proteins in reptiles, birds, and mammals and demonstrate that many have arisen independently in these lineages. Clusters of PRY-SPRY TRIM genes have undergone expansion and contraction in the avian MHC region and in the TRIM25 locus. We analyzed RNA-seq data from ducks infected with either highly pathogenic or low pathogenic avian influenza. We found many MHC-linked TRIM genes upregulated in response to highly pathogenic virus, and TRIM25 locus genes upregulated by both influenza infections. To assess for antiviral activity, we overexpressed candidate TRIM proteins and challenged with low pathogenic influenza virus and assessed infection by flow cytometry. Some TRIM proteins have antiviral activity against influenza A virus. Evolution and expression patterns of the TRIM repertoire suggests some are also likely targets of viral subversion.

Investigating the molecular and functional evolution of the CD300 family of innate immune receptors

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Vertebrate genomes encode numerous clusters of highly similar genes that arose through successive gene duplication events. Many of these clusters encode innate immune receptors that recognize pathogens and infected cells. Several of these immune receptor families exhibit dramatic variation due to gene gain and loss events across vertebrate lineages. For example, the clustered CD300 gene family encodes innate immune receptors, but the number of genes within the cluster varies between species. Because CD300 proteins have been shown to directly bind phospholipids implicating them in the recognition of non-self, they provide an exceptional model for studying the molecular and functional evolution of orthologs and paralogs within a clustered gene family. Here we outline the initial steps in utilizing a new multi-omics approach to 1) define the mechanisms of interspecific CD300 gene cluster expansion and diversification, 2) determine how interspecific diversification impacts extant innate immune receptor function, and 3) experimentally test how ancestral changes shaped extant functional diversity. This project will span all major (>260) vertebrate lineages, providing the most comprehensive metaanalysis of ortholog/paralog functional evolution within immune gene clusters. The results will illuminate the role of paralogs as a substrate for neofunctionalization and establish a foundation for predicting the association of immune gene clusters with immune function and disease susceptibility.

Characterization of An Ancient MHC-linked Nonrearranging Antibody and NK Receptor in Shark

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Gnathostome adaptive immunity is defined by the antigen receptors (immunoglobulins (Ig) and T cell receptors (TCR)) and the major histocompatibility complex (MHC). Evolutionarily, these hallmarks of adaptive immunity appear at the base of the vertebrate tree, in cartilaginous fish, and therefore examining the shark immune system has been crucial to elucidate the origins of adaptive immunity. We and others had hypothesized that ancestors of antigen receptors were encoded in the MHC precursor region and shaped as vertebrate genomes duplicated and then modified. Consistent with this concept, we and others have discovered nonrearranging antigen receptor-like genes in several vertebrates, many of which are encoded in the MHC or MHC paralogous regions. Those genes with nonrearranging variable (V) domains can be candidates for the precursors of antigen receptors and thus studying those genes will likely reveal some ancient functions of antigen receptors. Previously, we discovered that the nonrearranging V-containing NK receptor, NKp30, is the only evolutionarily conserved natural killer receptor among vertebrates, and proposed that characterizing shark NKp30 will reveal ancient function of NK receptors. More recently, we found an an ancient Ig-like gene, UrIg, with typical V- and Constant (C)- domains, like those found in conventional Ig/TCR, in the shark MHC. These nonrearranging genes likely predated the invasion by the RAG transposon and thus the presence of these V genes in the cartilaginous fish MHC is consistent with theories regarding the emergence of Ig/TCR within the proto-MHC. We will further discuss genome evolution of antigen receptors and the evolutionary significance of these genes in vertebrate immune system.

Evolution of a histocompatibility locus in basal chordates

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Multiple species across the tree of life can distinguish related and unrelated tissues and organs within the same species. *Botryllus* is a colonial tunicate where this allorecognition phenomenon is observed as two phenotypes: fusion and rejection. Allorecognition in *Botryllus* is controlled by a single highly polymorphic locus known as *fuhc* (**fu**sion/**h**isto**c**ompatibility), where six allorecognition genes have been isolated. However, it has not been established how these genes have evolved in tunicate species. We used transcriptomic and genomic data to understand the evolution of the *fuhc* locus. We found that this locus was assembled in a stepwise manner, where allorecognition genes appeared as non-polymorphic genes and subsequently acquired polymorphism. These results offer a unique opportunity to observe how a histocompatibility locus was assembly though evolution, which can not be observed at the same resolution level with the MHC complex of vertebrates. On the other hand, previous studies have shown that allorecognition in *Botryllus* is controlled by two receptors (fester and uncle fester). Using transcriptomic, genomic, and PCR data, we established that these receptors belong to a gene family with at least 40 *fester* members in *Botryllus*. These receptors exhibited different molecular mechanisms to generate variability. Moreover, we identified a new family of receptors with tyrosine-based motifs in the cytoplasmic regions, which were called *fester-coreceptors*. We found that each *fester* gene is linked to a *fester-coreceptor* gene in the ascidian genomes, exhibiting similar mechanisms of variability. These findings illustrate that allorecognition in *Botryllus* is controlled by multiple immune receptors, which share characteristics with allorecognition receptors of vertebrates.

SPLASH: a statistical, reference-free genomic algorithm unifies biological discovery

Julia Salzman, Stanford University.

Experimental and genomic strategies to characterize lymphocytes in the jawless vertebrates

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Adaptive immunity in the jawed vertebrates relies on complex mechanisms for somatic receptor diversification and intricate cellular selection systems. These systems are restricted to vertebrates and their evolutionary origins are unclear. In jawed vertebrates, adaptive immunity is centered around T cells and B cells which bear clonal immunoglobulin-based receptors. In the cyclostomes, the hagfishes and lampreys, a system of leucine-rich repeat (LRR) proteins, the variable lymphocyte receptors (VLRs), perform an analogous function. The VLRs are structurally unrelated to Ig and TCR but are nonetheless functionally rooted in similar lymphocytes. The extent to which the jawed and jawless vertebrate systems share an underlying regulatory structure is under investigation, but the distinctly different genomic histories and wide phylogenetic separation between these vertebrate groups present a unique window into early vertebrate immunity. I will discuss how we are using findings from single-cell RNA sequencing, comparative genomics, and experimental perturbation of the VLR systems to characterize the functional and evolutionary relationships of lymphocytes in these two vertebrate lineages.

Conservation of cytokines in echinoderms and other invertebrates

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Invertebrates rely on innate defense mechanisms to protect themselves against infections by pathogens. Cytokines mediate the cell to cell communication that is critical to coordinate cell migration and the release of immune effectors. This in turn results in the killing or eviction of these microbes from the host. While the factors involved in these processes have been studied in depth in mammalian models, far less is known about them in invertebrates with the cytokines and their receptors remaining poorly if at all characterized. Here we report our efforts on characterizing interleukin-17 and other putative cytokines in a range of invertebrate models.

Ruptoblasts act as surveillance for hormonal dysregulation in planarian

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Hormones are crucial regulatory molecules of various physiological processes. In mammals, many key hormone precursors are recognized by T-cells as auto-immune antigens, providing a surveillance mechanism to prevent overproduction of hormones and eliminate hypersecreting clones. However, it remains unknown whether such immunological regulation of hormones is conserved in other animals, especially those lacking adaptive immunity. Here, we report that surgically stitching together tissues from two genotypes of planarian flatworm, *Schmidtea mediterranea*, induces aberrant activation of activin signaling, which causes tissue degeneration mediated by p38. We identify a specialized cell type responsible for this inflammatory response to activin, which we term ‘ruptoblast’. Upon activin stimulation, ruptoblasts rupture and explosively release their granular cytoplasmic contents, killing adjacent cells. These ruptoblasts are found in close vicinity to activin-secreting intestinal cells and stem cells, which can generate more intestinal cells. Therefore, ruptoblasts can help to maintain stringent control over activin levels by eliminating secreting cells and their progenitors if local activin concentration becomes too high. Given that activin is a key hormone in regulating regeneration, homeostasis, and reproduction in planarians, our results suggest that, even in the absence of adaptive immunity, animals can evolve strategies parallel to T-cell surveillance to monitor hormone levels.

A limbic immune system of vertebrates

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Innate/innate-like lymphocytes are unconventional lymphocytes that do not fit the traditional innate and adaptive immune system paradigm. Whilst they originate from common lymphocyte precursors, they diverge early and mature by mechanisms employing PLZF as a lineage specifying transcription factor. Hence, innate/innate-like lymphocytes are distinct from innate immune cells of myeloid lineage as well as conventional B & T lymphocytes. They home strategically to non-lymphoid, barrier tissues for border patrol. Innate/innate-like lymphocytes function at the edge (limbus in Latin) of the innate and adaptive immune systems and, thereby, appear to form a 'limbic immune system'. In this proposal for a triune immune system—the innate, limbic & adaptive, we make no assumption that the 'limbic immune system' is an evolutionary transition between the innate and adaptive immune systems. But instead, the 'limbic immune system' is a conglomeration of independently acting immune effector modules, arising at different times in evolution, in many instances, repurposing loosely common genome regulatory circuits to accomplish a common task: to integrate information relayed by the innate sensory immune system about the local tissue umwelt and to provide context to downstream effector innate and adaptive immune responses. The multiple modules add robustness and evolvability to this limbic system to keep abreast of the ever-changing environment and the quick-evolving microbial cosmos, especially of those members of an otherwise symbiont community that turn pathobiont without much notice!

The fishy origins of germinal centers

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Germinal centers (GCs) or analogous secondary lymphoid microstructures (SLMs) are believed to have emerged in warm-blooded species. Thus, for decades the outstanding question has remained as to how and where adaptive immune responses are induced in cold-blooded vertebrates in the absence of GCs or analogous SLMs. Here we show that upon infection of rainbow trout (a teleost fish) with the parasite *Ichthyophthirius multifilii*, large aggregates of highly proliferating IgM^+ B- and $CD4^+$ T cells are induced contiguous to splenic melanomacrophage centers (MMCs). Most of these MMC-associated lymphoid aggregates (M-LAs) contained numerous antigen (Ag)-specific B cells. Analysis of the IgM heavy chain CDR3 repertoire of microdissected splenic M-LAs and non M-LA areas from these fish showed that the most frequent B cell clones induced upon the response were highly shared only within M-LAs of infected animals. These analysis also showed that M-LAs represent highly polyclonal SLMs in which Ag-specific B cell clonal expansion occurs. M-LA-associated B cells expressed high levels of activation-induced cytidine deaminase and underwent significant apoptosis. Critically, we found that somatic hypermutation of Igm genes occur for the most part in M-LA associated B cells. Recent data also shows that upon infection, the B cell compartment of the overwhelming majority of induced M-LAs is composed of IgM^+ B cells rather than IgT^+ B cells. These data is in line with the very low levels of IgT reported in serum when compared to those of IgM. In conclusion and contrary to current dogma, we show that ectotherms evolved organized SLMs with GC-like functions. Our data suggests that in addition to inducing systemic antigen-specific IgM responses, M-LAs are also involved in the generation of IgM responses against microbiota. In addition to unveiling the most ancient SLMs described thus far in vertebrates, our results also point to primordially conserved mechanisms by which fish M-LAs and newly discovered polyclonal GCs of mammals develop and function.

Are canonical dendritic cells the original "follicular dendritic cells?"

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After immunization to a foreign protein in adjuvant, *Xenopus* XL cells bear the native antigen in the splenic white pulp. Previously XL cells were shown to be class II-high, bear all immunoglobulin isotypes on the cell surface, and express the canonical hematopoietic transcription factor PU.1. We now show that XL cells also express CD45 and transcriptome analysis demonstrate that XL cells are of the myeloid lineage. White pulp XL cells are non-specific esterase-negative, and are weakly adherent and phagocytic. These data confirm that XL cells are most like mammalian dendritic cells. Because XL cells do not migrate to splenic WP post-immunization in T cell-deficient frogs, we have proposed that they must be licensed by T cells to perform this function after XL cells present MHC-restricted antigen to T cells; thus, XL cells have been dubbed 'Double-duty dendritic cells (DDDC).' We examined XL cell phenotype movement every day post-immunization (D0-15). By FACS, XL cell numbers do not change over the course of the immunization, but their migratory patterns in the spleen clearly change over time. XL cells are present in the WP and red pulp (RP) at D0, but appear to migrate out of the WP early after immunization coincident with a breakdown of the Border of Sterba that surrounds the WP. We propose that early in the response XL cells present antigen to T cells in the RP. Antigen-laden XL cells are found in the WP at D8 and then abruptly in their typical oval arrangement in the WP at D9. Plasma cells are detected as early as D12, followed by a second disruption of the WP starting at D20 and an apparent efflux of the XL cells. Previously we demonstrated that a "T cell zone" forms around the WP at ~D9, and this structure persists for up to 60D post immunization; in unimmunized animals T cells are randomly scattered throughout the RP. Matz et al found a somewhat similar kinetics of antigen movement into nurse shark WP after immunization, with a coalescence of T cells around the WP. We have confirmed their data and found the antigen in the center of very large white pulps post immunization, as well as the appearance of antigen-positive cells around the WP. We also confirmed the coalescence of T cells around the WP. We have detected the antigen-laden cells by FACS and hope to perform transcriptome and functional analyses. In summary, there may be a general strategy for generation of humoral responses in ectotherms prior to the emergence of follicular dendritic cells with the central player being the DDDC.

Xenopus myeloid cells: Identification with monoclonal antibodies and the binding of Ig isotypes

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There is much to be learned about the function of myeloid cells in ectothermic vertebrates. Our previous work on *Xenopus* dendritic-like cells, so-called XL cells, showed that they are MHC class II-hi, present native antigen in the *Xenopus* splenic white pulp (WP), and bear the three immunoglobulin (Ig) isotypes, IgM, IgX, and IgY on the cell surface. We now show that peritoneal macrophages (PEC) are also class II-hi and bear all three Ig isotypes, but unlike XL cells they are highly phagocytic and non-specific esterase-positive. PEC also have a transcriptome like mammalian macrophages. We examined other myeloid populations after immunization with phycoerythrin in incomplete Freund's Adjuvant. Eosinophils in the blood and spleen are class II-lo and bear Igκ but not IgY. Neutrophils in the blood are class II- and IgY-negative and also bear Igκ. Monocytes express class II at the same level as B cells and bear IgY but not IgM/IgX. Splenic red pulp macrophages are strongly non-specific esterase-positive, but preliminary evidence suggests they do not bear surface Ig. After immunization, the PEC are not present in the peritoneal cavity, "replaced" by an influx of neutrophils. Finally, we detect changes in the neutrophil population over time such as no Igs on the cell surface, presumably because immature cells arising from the bone marrow are FcReceptor-negative. Several previously poorly characterized mAbs were shown to delineate the different myeloid (and lymphoid) populations. These data will be instrumental in parsing the functions of different *Xenopus* hematopoietic subpopulations.

Evaluation of Recombinant *Flavobacterium covae* Protein Vaccines in Channel Catfish *(Ictalurus punctatus)*

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Columnaris disease is one of the leading causes of mortality in channel catfish production. *Flavobacterium covae*, an etiological agent of columnaris disease, has shown to be highly virulent in channel catfish and is a major problem for the US aquaculture industry. Here, we test the immunogenicity and efficacy of several biofilm-associated *F. covae* proteins to be used as recombinant subunit vaccines. The genes encoding these predicted proteins were cloned into expression vector pET-28a(+). After expression in *Escherichia coli* strain BL21 (DE3), the antigens were purified under native conditions. Channel catfish were injected intraperitoneally with purified protein (20 ug/mL), and peripheral blood was collected 30 days post-vaccination. Preliminary data shows vaccinated fish exhibited sera IgM antibody specificity to the respective antigens when blotted to the reduced proteins and had an increase of sera IgM antibodies. In our next trial, groups of fish (n=540) were immunized by bath immersion with the recombinant protein(s) (1 ug/ml) or sham immunized. There was no significant mucosal IgM antibody production among the vaccinated groups. However, each vaccinated group showed significant survival when challenged with *F. covae* (>30% compared to the control group) at nine weeks post-immunization. Significant upregulation of innate and adaptive and immune genes was seen in vaccine groups compared to baseline gene regulation in the sham immunized group. The efficacy of the vaccine(s) delivered orally via top-coating feed will be discussed as well. These results lay the groundwork for potential vaccine candidates to protect farmed fish against columnaris disease during the production cycle.

Using a phytoglycogen nanoparticle to carry innate immune modulators to gut tissue in rainbow trout

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Rainbow trout are an economically important fish species that, when farmed, are highly susceptible to virus infections. Available vaccines are variable in their efficacy thus antiviral treatments, preferably delivered orally, could play an important role in protecting animals during their most vulnerable times, such as early in their development or during transport. In this study, innate immune stimulants were delivered to the gut of rainbow trout using a phytoglycogen nanoparticle (Nanodendrix, NDx) derived from sweet corn. Double-stranded (ds)RNA, a virus pathogen associated molecular pattern (PAMP), complexed with NDx was capable of inducing a robust type I interferon (IFN) response in rainbow trout gut cells (RTgutGC). This response was transmitted to rainbow trout monocyte/macrophage cells (RTS11) when the RTgutGC cells were grown in inserts with RTS11 on their basal surface. In vivo trials with dsRNA-NDx integrated into feed demonstrated induced IFN responses both in the gut and the head kidney indicating a local and systemic innate immune response to the dsRNA. CpG oligodeoxynucleotides (ODNs) have also been complexed to NDx and the NDx has enhanced CpG ODN delivery to RTS11, suggesting a combination of nucleic acids can be delivered using NDx. Protocols to optimize innate immune stimulation in rainbow trout gut perfusion experiments have been developed using lipopolysaccharide as an innate immune stimulant. Combined, this work demonstrates NDx is an effective carrier of innate immune stimulants to the gut and an excellent candiate for prophylactic antiviral treatments in fish.

Towards the Development of an In Vitro Research-scale Bioreactor System to Mass-cultivate Massive Numbers of Autologous Fish Antigen-Presenting Cells

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This research reports an *in vitro* approach to culture, propagate and mass-produce of antigen-presenting cells (APCs) for fish. Our current model species is rainbow trout, which represents one of the most important fish in Canada. Our primary interest is to establish a non-lethal method to culture and generate an autologous population of APCs for potential use in studying antigen presentation *in vivo*. Isolating and growing fish APCs isolated from peripheral blood faces challenges as their number is notoriously low in blood and there is no method to grow them. During our search for other tissue types as sources to support and generate APCs, we discovered tissue cultures with fin clipping explants proving to be incredibly fertile for trout APCs to thrive and multiple. After several months of *in vitro* incubation with a routine schedule of selective cell enrichment and specifically formulated nutrient feeding, we demonstrated that vessels of fin explant cultures could produce over a billion APC-like cells. These APC-like cells had morphologies that resembled mammalian DCs and had abundant expressions of major histocompatibility class II (MH II), CD83, CD209, and CD205 (markers of mammalian DCs). Adding exogenous zymosan or Vibrogen to the fin-derived APCs led to an increased level of IL-2 protein in the culture medium. As fin clippings do not threaten the survival of fish and fins are natural regenerative appendages, our method enables the generation of massive numbers of autologous fish APCs, which can be used to pursue new research questions, biotechnology innovations, and veterinary uses.

Examination of zebrafish (*Danio rerio*) leukocyte immune-type receptor-mediated crosstalk regulation of phagocytosis

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Immunoregulatory receptors play a crucial role in coordinating immune responses against microbial invaders by initiating intracellular signaling events. Leukocyte immune-type receptors (LITRs) are a diverse group of proteins found in fish that share similarities with the mammalian immunoglobulin superfamily (IgSF) members. Traditionally, immune-type receptors have been categorized as either stimulatory or inhibitory based on the presence of specific motifs within their CYT regions, known as immunoreceptor tyrosine-based activation motifs (ITAMs) or immunoreceptor tyrosine-based inhibitory motifs (ITIMs), respectively. Overall, LITRs regulate innate immune functions through their cytoplasmic tail (CYT) regions, inducing various intracellular signaling networks. When multiple immunoregulatory receptors interact with common ligands and recruit signaling molecules, it fine-tunes the overall response, and this phenomenon is known as receptor crosstalk.

The focus of my research project is to examine receptor crosstalk between two unique zebrafish (Dr) LITR-types using a flow cytometric-based phagocytic assay approach developed in our lab. I have developed a stably co-expressing cell line in AD-293 HEK cells through successful cellular transfection. This cell line expresses a putative stimulatory construct, DrLITR 1.2wt which consists of an ITAM and ITIM within the same CYT, while the CYT of DrLITR 15.1wt contains two ITIM motifs and an immunoreceptor tyrosine-based switch motif (ITSM) exhibiting inhibitory potential. Ongoing work is focused on optimizing conditions for co-engagement of mAbs with co-opsonized beads to examine phagocytosis by conducting meticulous comparisons of monoclonal antibody concentration on opsonized bead targets and bead-cell incubation times. This research underscores the significance of employing imaging flow cytometry as a valuable platform for exploring the dynamic signaling potential of DrLITR-types in regulating immune cell effector responses.

The characterization of phagocytic cells and the kinetics of acidification

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The American Cockroach (*Periplaneta americana*) provides an interesting model to study the innate immune system. In contrast to drosophila, the American cockroaches are hindgut fermenters with complex microbiomes like humans. This, coupled with the recent ability to produce axenic Cockroaches, allows us to study how the change in microbiome affects the immune system. However, we must first determine the specific functions of hemocytes within the hemolymph of the American cockroach. Here, we use pH-sensitive probes to track the uptake and acidification process. This, along with lectins, allows us to differentiate between cell types that can phagocytize. We have found, through the use of flow cytometry and microscopy, two distinct populations of cells that have phagocytic activity. Also, ongoing research using pulse-chase experiments shows that while the acidification of particles can happen as quickly as two hours post-treatment, the peak abundance of positive cells and mean fluorescence intensity (MFI) appear to be around eight hours after the initial pulse and a gradual decay up to 24 hours later. In vivo studies with these methods will hopefully shed light on the migration hemocytes to a wound or infection site. These experiments will allow us to identify which cells can phagocytize and the kinetics of phagocytosis in the American cockroach.

Deciphering the roles of CXCL8 chemokines in amphibian antifungal defenses

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The *Batrachochytrium dendrobatidis* (*Bd*) chytrid fungus is significantly contributing to the global amphibian declines. While *Bd* infections are confined to amphibian skin, considerably little is known about the amphibian skin-resident immune populations and their respective roles in *Bd* susceptibility and resistance. Our recent work indicates that *Xenopus laevis* frogs express notable levels of CXCL8a and CXCL8b chemokines in their skin, presumably homing select immune lineages therein. The *X. laevis* CXCL8a possesses an ELR motif characteristic of mammalian proinflammatory chemokines and appears to be involved in the frog inflammatory responses. Conversely, the *X. laevis* CXCL8b lacks this ELR motif and at least in some contexts recruits granulocyte-lineage leukocytes with an immunosupressive phenotype. While manipulating the levels of skin CXCL8a has no observed effect on *X. laevis* susceptibility to *Bd*, subcutaneously administering a recombinant form of CXCL8b to *X. laevis* renders them more susceptible to *Bd*. Conversely, depleting this chemokine from the frog skin renders the animals more resistant to *Bd* infection. Together, our findings suggest that CXCL8b and the immune subset(s) recruited into the frog skin by this chemokine are rendering frogs more susceptible to this fungal pathogen. We are presently exploring which leukocyte subsets are targeted by CXCL8b towards a more holistic perspective of amphibian skin immune defenses.

Analysis of developmental gene expression in the dorsal skin of North American wood frog (*Rana sylvatica*) larvae and metamorphs

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Amphibian skin is a vital immune organ, yet little is known about gene expression patterns in this tissue and how it changes during development. To address this, we leveraged transcriptomic data from North American wood frog (*Rana sylvativa*) dorsal skin tissue samples previously collected from a collaborative microplastics exposure experiment. Wood frogs were raised from wild-captured fertilized embryos in quadruplicate 300 L stainless steel outdoor aerated mesocosms containing 200 L of filtered (< 52 μm) lake water and either 0, 0.069, or 0.69 g/L of a microplastics mixture. Dorsal skin tissues were collected at three timepoints: Gosner stage (GS) of ~34; GS ~42; and at GS 45. Isolated RNA underwent polyA mRNA library preparation and paired-end Illumina sequencing. After aligning reads to the wood frog reference genome, StringTie was used to generate a transcriptome using all samples and discovered 96,645 transcripts belonging to 45,926 genes, of which 90,250 transcripts (belonging to 44,601 genes) were expressed in control (unexposed, 0 g/L microplastics) dorsal skin. DESeq2 revealed 6,730 genes are differentially expressed with a $\log_2$ fold change cut-off of 0.585 in at least one pairwise comparison between timepoints. Gene set enrichment analysis of GO terms and KEGG pathways reveals suppression of terms including those related to immune function, translation, RNA processing, mitochondria, and cellular signaling during development, along with activation of terms related to keratinization, transmembrane transport, epithelia, antigen presentation, T cell function, and GTPase. These results suggest dynamic regulation of immune function in the skin during amphibian development and support previous observations of immunosuppression during metamorphosis. [Funded by Environment and Climate Change Canada and NSERC Discovery grants].

Characterization of frog virus 3 infection in adult wood frogs (*Rana sylvatica*) following water bath exposure

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Frog virus 3 (FV3; Faimly *Iridoviridae,* Genus *Ranavirus*) is a causative agent of ranavirosis in many amphibian species worldwide and is one of the infectious diseases believed to be contributing to amphibian declines. The North American wood frog (*Rana sylvatica*) is susceptible to FV3 and infection can lead to mortality of juvenile and adult life stages. To begin to understand host-virus interactions in adult wood frogs, the objective of this research was to establish a water bath infection protocol. Adult male wood frogs were held in a 50 mL water bath containing 0, 10^2, 10^3, or 10^4 plaque-forming unit (pfu)/mL of FV3 for 3 hours prior to transfer to a terrarium. Over 11 days post infection (dpi), clinical signs of infection, including redness, petechiae, and low activity levels, were observed in wood frogs exposed to 10^3 or 10^4 pfu/mL of FV3. No mortality was observed in any group over the course of the study. To evaluate infection prevalence and intensity/viral replication, frogs were euthanized at 4 dpi (0, 10^4 pfu/mL, n = 3/group) or 11 dpi (0, 10^2, 10^3, 10^4 pfu/mL, n = 5/group) and tissues collected for downstream quantification of FV3 copy number per nanogram of genomic DNA. All doses of FV3 exposure resulted in 100% infection prevalence at 11 dpi, with levels of FV3 genome copies detected in tissues tending to increase in an exposure dose-dependent manner. Our findings demonstrate water bath exposure to FV3 can lead to systemic infection of adult wood frogs, and provides supporting evidence of the high susceptibility of all wood frog life stages to environmental transmission of FV3. Future RNA-sequencing studies will provide an understanding of the temporal antiviral immune responses of wood frogs during FV3 infection. [Funded by NSERC DG]

BHF, the *Botryllus* Gene that Predicts Fusion Rejection Outcome via Allelic Polymorphism, Aligning with Mendelian Inheritance of This Trait

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The major histocompatibility complex (MHC), a set of cell surface molecules encoded by a large gene family, was discovered through its role in the rejection of transplants. It has been shown that MHC controls a major part of the immune system in all vertebrates and determines self from non-self. In *Botryllus schlosseri*, a marine organism closely related to vertebrates, the decision to reject or fuse is governed by a different mechanism through a single polymorphic histocompatibility gene. A candidate gene called *FuHC* was first identified as the *Botryllus* histocompatibility gene (De Tomaso et al., 2005), but it was later found to be inconsistent with the expected genetic outcomes. Following the sequence and assembly of the Botryllus genome, a gene called *BHF*, which is located very close to *FuHC*, was then found to be a better candidate for the Botryllus histocompatibility gene (Voskoboynik et al. 2013a; 2013b). *BHF* mRNA sequence perfectly predicts fusion rejection outcomes, matches heterozygosity/homozygosity lines and self-BHF recognition inhibits cytotoxicity, whereas non self BHF recognition induces killing (Voskoboynik et al. 2013a; Rosental et al. 2018). It does not have any homologues in vertebrates based on its nucleic acid sequence, amino acid sequence, or predicted structure. It also does not have any motifs to indicate that it is a membrane-bound or secreted protein by classical criteria. However, BHF is expressed on the cell surface, which suggests that it may have a unique mode of action. Here I'll review the pathway that led to the discovery of BHF and its connection to FuHC, and will discuss the current knowledge surrounding these genes.

The purple sea urchin response to bacterial challenge examined via single cell nuclei RNA-Sequencing

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Echinoderms offer a unique perspective on the evolution of immune systems. As an invertebrate deuterostome, echinoderms can provide insight into the earliest evolutionary steps towards the origins of vertebrate adaptive immunity. One of the best-characterized echinoderm immune systems is that of the purple sea urchin (*Strongylocentrotus purpuratus*). The purple sea urchin has a biphasic lifecycle that includes a planktonic larval stage. Larvae respond to specific pathogens by activating a robust cellular and transcriptional immune response. To characterize this immune response at single-cell resolution, *S. purpuratus* larvae were exposed to the marine bacterium *Vibrio diazotrophicus* and used in single-nucleus RNA-Seq (snRNA-Seq). Extracting nuclei allows us to specifically quantify genes that are actively transcribed during this immune response. Results have identified ten specific cell clusters that variably respond to bacterial infection. This work sheds light on how the system-wide larval immune response is coordinated and how specific cell types contribute to this response.

Molecular characterization of Botryllus schlosseri circulating blood cells based on single-cell RNA-seq analysis

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Circulating blood cells in the colonial ascidian *Botryllus schlosseri* are key players involved in diverse immunobiological and homeostatic processes. We carried out scRNA seq of circulating blood during two stages of asexual development. Analysis of differentially expressed immune-related genes shows that the circulating cells are more diversified than previously recognized. While some populations showed partial overlap with previously reported characterizations, many were unique. For the former, a population of cells were identified that were expressing high levels of the allorecognition protein, fuhc-sec, which had been described previously. Analysis of the expression of various innate immune genes, including pattern recognition receptors and their associated signal transduction molecules, IL-17 and IL17R, 'eat-me' receptors and other phagocytic markers, as well as prophenoloxidase show unexpected diversity in putative innate immune cells. In addition, several of these populations were restricted to one or the other stage of asexual development, and germline stem cells were only observed in circulation during mid-cycle, as had been described previously. We are currently using in situ hybridization to correlate these populations with cellular morphology. Current results will be discussed.

Functional And Molecular Characterization Of Germline Stem Cells In A Marine Tunicate And Their Role In Gonadal Regeneration

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Botryllus schlosseri is a marine tunicate which undergoes a weekly stem-cell-mediated whole body regeneration process. While various types of stem cells regenerate different tissues, only germline stem cells (GSCs) will establish the gonads and pass their genes to the next generation via sexual reproduction.

To isolate GSCs and explore their fitness, testes were dissociated from various developmental stages, cells were stained with non-species-specific stem cell markers and analyzed using flow cytometry. Based on morphological parameters, different stages of cells along the spermatogenesis process were separated. While spermatozoa and spermatid stages were easy to identify based on morphology, GSCs were impossible to tell apart. Therefore, cells were index-sorted followed by single cell RNA sequencing using the SMART-Seq III pipeline. Analysis of the cell clusters revealed expression of homolog genes to germline cell markers, as also proved by *in-situ* hybridization of those genes in *Botryllus* testes. By employing index-sorting, we could associate the transcriptomic fingerprint of potential GSCs with their positions on the FACS plots and sort those specific cells for further functional assays. Subsequently, these cells were sorted and transplanted into reproductive colonies. A week after transplantation, the cells were tracked *in-vivo* and discovered within the testes of the recipients. Some of the labeled transplanted cells underwent differentiation into spermatozoa, indicating that the transplanted cell population was enriched with GSCs.

The isolation of these cells marks a pivotal step in unraveling the factors that govern gonadal regeneration and how the fitness of germline stem cells changes as an animal ages.

Single-nuclei transcriptome analysis of channel catfish IgM-positive splenic fractions provides insight into the fish immunome from an aquaculture-relevant species

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The catfish industry is the largest sector of U.S. aquaculture production. Channel catfish, *Ictalurus punctatus*, is an important model for studying teleost fish immunity. Given its role in food production, the catfish immune response to industry-relevant pathogens has been extensively studied. To further examine the channel catfish immune system, we performed single-nuclei RNA sequencing on IgM-positive splenic B-cells (n=3). Spleen cell suspensions were passed through a cell sieve. To isolate IgM-positive B-cells, spleen lymphocytes were labeled with a recombinant monoclonal antibody that is reactive to catfish IgM and sorted using flow cytometry. Single-nuclei RNAseq libraries were then prepared using the 10X Genomics platform and sequenced on an Illumina NovaSeq X Plus. The demultiplexed samples was aligned to the CoCo_2.0 channel catfish reference assembly and filtered using Cell Ranger (v.5.0.0). Integrated data were analysed in Seurat (v.5.0.1) and trajectory analysis was carried out using with velocyto (v.17.17) and scVelo (0.2.5). The fractionated B Cell samples generated a total of 753,493,178 reads from an estimated 21,776 cells with approximately 34,602 reads/cell. 67% of reads mapped confidently to approximately 22,822 genes which equated to approximately 1,048 genes/cell. Preliminary analysis has identified 14 clusters in the aggregated data and 12 clusters expressed B cell markers. These data will create a profile of B cell subsets to a high-resolution in channel catfish and will provide crucial information on innate and adaptive immune function during disease progression.

The single-cell transcriptome analysis of channel catfish B and T cell lines

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Channel catfish (*Ictalurus punctatus*) have been shown to be an excellent model for understanding teleost immunity, and the development of several cell lines has proved to be indispensable in many fundamental studies into the function of B and T lymphocytes. There are two B cell lines (1G8 and 3B11) developed through *in vitro* LPS stimulation and two T cells lines, one developed through *in vitro* LPS stimulation (G14D) and the other (28S.3) was spontaneously immortalized after the long-term culture of peripheral blood lymphocytes. All cell lines were cultured at 27°C, 5% CO_2 in AL-4 medium consisting of equal parts of AIM V medium and L-15 medium adjusted to catfish tonicity with 10% DI H_2O and supplemented with 1 mg/mL $NaHCO_3$, Penicillin Streptomycin Glutamine solution, 50 mM 2-ME, and 4% heat-inactivated pooled catfish serum. $2.0x10^4$ cells (~90% cell viability) for each were loaded onto a GEM-X chip and single-cell RNAseq libraries were prepared using the 10X Genomics Chromium platform and later sequenced (~$2.0x10^4$ reads/cell) on an Illumina NovaSeq X Plus. The demultiplexed samples will be aligned to the CoCo_2.0 channel catfish reference assembly and filtered using Cell Ranger (v.5.0.0). Integrated data will be analyzed in Seurat (v.5.0.1). Cluster analysis will be performed to identify any heterogeneity among the different cell lines. Differential gene expression and markers that define individual cell types will be discussed.

Macrophages at the center of amphibian antifungal defenses and targets of fungal counter defenses

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Amphibians continue to experience global population declines due to chytridiomycosis caused by the chytrid fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). Although amphibians have an arsenal of robust immune defenses, this pathogen appears to have evolved counter defenses that permit it to survive in amphibian skin. Previous studies have demonstrated that live or dead *Bd* cells or cell-free supernatant factors directly inhibited lymphocyte function by induction of apoptosis suggesting a mechanism for impairment of local immune cell killing. However, there is little evidence for lymphocyte recruitment to chytrid-infected skin suggesting that the fungus can also inhibit recognition and effector function of antigen presenting cells such as macrophages. The capacity of peritoneal leukocytes (PLs) enriched in macrophages (Mfs) and neutrophils to phagocytose pHrodo™ Green Zymosan BioParticles™ was significantly reduced by co-culture with live or heat-killed *Bd* cells. Cell-free supernatants produced by matrue *Bd* sporangia also inhibited uptake of pHrodo™ Red *Staph aureus* BioParticles™ in a dose-dependent fashion. Additional studies suggest that the phagocytic capacities of frog bone marrow-derived Mfs, differentiated by colony stimulating-factor-1- or interleukin-34 (key Mf growth factors) as well as a mammalian Mf cell line were also impaired by *Bd*. Together, our findings suggest that by suppressing phagocytosis, *Bd* may be subverting a critical processes needed for antigen presentation in the skin. Future work will explore the mechanisms and consequences of this inhibition to amphibian myeloid cell functionalities. Support: NSF 2147466 (LG) and 2147467 (to LR-S).

Exploring the functionalities of amphibian mast cells

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The *Batrachochytrium dendrobatidis* (*Bd*) chytrid fungus infections of amphibian skin are significant drivers of the global amphibian declines. Unfortunately, amphibian skin-resident immune populations have been relatively understudied. This knowledge gap has obscured our perspectives of amphibian antifungal defenses, consequently hampering conservation efforts. Pertinently, our recent studies indicate that frog (*Xenopus laevis*) mast cells are integral to skin antifungal defenses, although the mechanisms of frog mast cell anti-*Bd* protection are not fully understood. Presently, to elucidate amphibian mast cell immune capacities, we developed methods for establishing *X. laevis* mast cell cultures from peritoneal (PMCs) and bone marrow-derived (BMMCs) precursors. Mammalian mast cells mediate many of their effects by releasing preformed immunomodulatory compounds, predominatly studied in the context of IgE-mediated degranulation. As amphibians lack IgE, we explored alternative immune stimuli that may trigger degranulation of *X. laevis* PMCs and BMMCs, and analyzed the contents of these degranulates. Together, our studies represent an important step towards a greater understanding of amphibian mast cell functionalities and highlight how these cells may contribute to protection against *Bd* infections.

Classical techniques in an unconventional system: Evaluation of the American Cockroach immune system from a flow cytometric perspective

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Insects, such as *Drosophila,* have served as a valuable model for immunological studies, and in recent years, there has been a growing interest in other insects for immunological studies. The American Cockroach *(Periplaneta americana)* has a complex gut microbiome compared to other insects, mirroring the microbial diversity found in mammalian guts. Recently, protocols have been generated to hatch axenic cockroaches. These characteristics stage *P. americana* as an exciting model for investigating the impact of the host microbiome in innate immune development and function. However, the lack of an annotated genome, conventional reagents (e.g., antibodies), and standardized evaluation of insect immune cells (hemocytes) limits these research avenues. To address these barriers, we employ flow cytometry in combination with lectins, lysosome indicators, and reactive oxygen species indicators to investigate *P. americana* hemocytes. These reagents provide a replicable means of identifying and sorting cockroach immune cells. Additionally, we identified candidate immune genes through sequence homology via BLAST to develop primers for RT-qPCR. Through a combination of RT-qPCR for immunologically significant genes and cell sorting, we can better connect cell function to cell identity. These findings lay the necessary groundwork for our future analyses of axenic cockroaches.

Darkest Before Dawn: Cellular and Kinetic Characteristics of Melanization in the American Cockroach

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Insects, such as *Bombyx mori* and *Drosophila melanogaster* are important models in immunological studies. The American cockroach (*Periplaneta americana)* is another intriguing model for immune studies, as its gut microbiota has a complexity like humans, and recent protocols enable the production of germ-free cockroaches. However, despite critical avenues of research presented by this organism, investigation of its robust immunological landscape has paled in comparison to that of contemporaries, leaving a knowledge gap in our baseline understanding of insect immune processes carried out by hemocytes (insect immune cells). A particularly unique immunological mechanism in many invertebrates is melanization. Beginning with the production of extracellular reactive oxygen species and ending with the synthesis of pigmented cytotoxic melanin, this mechanism reduces the viability of pathogenic organisms. Identification of the cellular subsets and components contributing to the melanization reaction in the American cockroach is instrumental to understanding this immunological landscape. Using classical immunological and molecular techniques such as cell sorting, brightfield microscopy, and spectrophotometry, we utilized functional assays to identify the cellular and kinetic characteristics of the melanization reaction in the American cockroach. In our studies, we identified two hemocyte subsets responsible for hemolymphatic melanization, with spectrophotometry dictating that one subset possessed the fastest kinetic rate of this reaction. With this foundational knowledge, we aspire to establish a link between innate immune system development and the gut microbiota, using the American cockroach as an inexpensive model for studying this commensalism. Additional studies include validation through RT-qPCR of cell types for genes involved in melanization.

Functional study of the endogenous antigen presentation pathway in Rainbow trout

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One of the key processes of adaptive immunity is the antigen presentation pathway which has been extensively studied in higher vertebrates. Previous studies have shown that the transcripts and protein levels of *mhc-i*, *mhc-ii, tapasin, beta 2-microglobulin,* among others*,* are impaired under different stimuli such as suboptimal temperatures and pathogen challenges. We focused on the function of the MHC-I molecules and ER chaperones such as b2m, tapasin and ERp57 at suboptimal temperatures after an infection of the VHSV in RTS-11 and RTGut. Cell surface expression of the MHC-I/B2m complex was evaluated by flow cytometry obtaining three different cell populations: MHC-I/b2m double positive cells, cells expressing MHC-I alone (Free MH-I), and cells negative for both MHC-I and b2m (double negative cells). The expression of MHC-I/b2m complex decreases at higher temperatures over time, however, at 4C the levels of MHC-I/b2m positive cells are over 50%, increasing to 80% in infected cells at 4dpi. Starting at 4dpi there is an increase of double negative cells in the infected group at 14C, suggesting that VHSV may decrease antigen presentation capacity in RTS-1. In RTGut, there is a slight increase of double negative cells at 9dpi in the infected group at 14C, reaching 5% of the total population. There is an increase in proteasome activity at 9 dpi at 14C in RTS-11 cells. Finally, the secretion of the key antiviral cytokine IFN-1 was evaluated by sandwich ELISA, and as expected there is an increase of IFN-1 secretion after the infection with VHSV at 14C and 20C but not at 4C, in both RTS-11 and RTGut. Thus, rainbow trout could adapt to suboptimal temperatures by having a functional antigen presentation pathway. In addition, we are developing an antigen presentation assay by utilizing dorsal fin cells as professional antigen presenting cells.

Allelic diversity of TAP and tapasin in wild mallards in Alberta, Canada

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Antigen presentation is a key element for an appropriate immune response to pathogens and tumors threatening healthy organisms. We previously showed that in wild mallards only one polymorphic MHC class I gene (UAA) is predominantly expressed, and it is the one adjacent to the TAP2 gene which has not been studied in terms of diversity. Chickens have a minimal essential MHC where MHC class I, TAP and tapasin genes are in very close proximity making recombination difficult and resulting in haplotypes expressed as a cassette. These genes have coevolved to have the perfect antigen fit allowing efficient antigen presentation by maximizing the peptide affinity to MHC class I. It is unclear whether this is also true for ducks. Using PCR amplification from genomic DNA and RNA transcripts and sequencing, we analyzed the differences of expressed TAP and tapasin alleles of 13 mallards, the natural reservoir of influenza virus. We found that TAP2 is very diverse and polymorphic in ducks contrary to TAP1 and tapasin, which have mostly dimorphisms. Many of the polymorphisms found in TAP2 are located in regions involved in the peptide binding in mammals, whereas only 2 aminoacid substitutions were found in the TAP1 peptide binding region. We predict that haplotypes are stable, however, conflicting data leaves this unresolved. Our findings suggest that TAP2 diversity contributes to the peptide repertoire presented to MHC class I and may limit the adaptive immune response to influenza.

Examining rainbow trout vig-3 expression patterns, induced expression and antiviral roles following dsRNA treatment

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Interferon-stimulated genes (ISGs) are key mediators of antiviral immunity. Viral hemorrhagic septicemia (VHSV) – induced gene 3 (vig-3) is a highly inducible, multi-functional ISG in rainbow trout whose expression patterns, induction, and antiviral role remain relatively unknown. In this study, vig-3 transcript expression was identified in a panel of tissues from healthy rainbow trout. Vig-3 transcripts were upregulated in response to recombinant type I interferon (IFN-I) treatment in rainbow trout gonadal cells (RTG-2). Vig-3 transcript and protein levels were upregulated overtime following treatment with polyinosinic: polycytidylic acid (poly IC), and infection with two viruses, infectious pancreatic necrosis virus (IPNV) and viral hemorrhagic septicemia virus (VHSV) in two rainbow trout cell lines, RTG-2 and rainbow trout gill cells (RTgill-W1). Western blot analysis demonstrated evidence of protein ISGylation, a unique feature of vig-3. Vig-3′s cellular localization was assessed using immunocytochemistry following viral infection. Vig-3 expression was cytoplasmic and increased in quantity over time, upon treatment with poly IC, or infection with IPNV or VHSV. The ability of novel phytoglycogen-based nanoparticles to enhance dsRNA-induced vig-3 and up-regulate inflammatory markers including type I interferon (IFN-I), interferon gamma (IFN-γ), and interleukin 1beta (IL-1β) was then assessed in RTG-2, RTGill-W1 and in a rainbow trout monocyte/macrophage cell line (RTS-11). The nanoparticles induced significant IFN-I and vig-3 expression at the transcript level in all cell lines, surpassing the effects than poly IC alone over time. These findings improve our understanding of the rainbow trout innate immune system and could aid in the production of fish antiviral therapeutics in the future.

Duck RIPLET activation of RIG-I and Influenza NS1 antagonism

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The Nonstructural Protein 1 (NS1) of influenza A viruses (IAV) has two main ways to suppress the host innate immune responses in human and mouse cells: downregulation of host protein synthesis through interaction with Cleavage and Polyadenylation Specificity Factor 30 (CPSF30) and disruption the RIG-I pathway by interacting with RIPLET which is an obligatory ubiquitin ligase essential for RIG-I activation and signaling. No studies have explored the functions of RIPLET and potential interactions with NS1 in avian species like the mallard duck, the natural reservoir of IAV. In this study, we investigate the role of duck RIPLET in RIG-I signaling and potential antagonism by NS1. We expressed NS1 proteins from different IAV strains together with duck RIG-I and RIPLET in chicken DF-1 cells, naturally devoid of endogenous RIG-I and RIPLET. Our findings demonstrate that wildtype NS1 proteins inhibit interferon signaling, however it is unclear whether this is through interaction with RIPLET or CPSF30. G184R mutants of NS1 that cannot bind CPSF30, fail to inhibit RIPLET-activated RIG-I signaling in avian cells. In contrast, these mutants downregulate RIG-I signaling in human cells. This research suggests a potentially unique relationship between duck RIPLET and NS1, revealing crucial insights into species-specific viral evasion mechanisms and highlighting the importance of comparative immunology in understanding host-pathogen interactions.

CD1-like, lipid-presenting, nonclassical MHC molecules in shark

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While CD1 molecules were originally predicted to have an ancient origin, *CD1* is absent in amphibians and bony fish and was thus thought to have first emerged in reptiles. We now report two MHC-linked, single- or low-copy MHC-Ib genes in sharks, *UFA* and *UGA*, which are unexpectedly more *CD1*-like than any other MHC gene. Both elasmobranch UFA and UGA, but not other shark non-classical class I molecules, cluster with amniote CD1 molecules based on biophysical properties, suggesting that they may binds lipids. Strikingly, crystal structures of both UFA and UGA shows them to have narrow, extremely hydrophobic grooves, like CD1 but not classical MHC-Ia. The antigen-binding groove of UFA is extrememly narrow/collapsed and ostemsibly empty in the crystal structure, although under certain *in vivo* conditions, it may open and allow lipid access to the hydrophobic interior. The antigen-binding groove of UGA, by contrast, has a large, deep pocket with two parallel grooves, containing long lipid chains. Mass spec characterization of lipids associated with UGA identified several lipid species frequently shared with those from human CD1d, confirming that UGA is also a lipid-presenting nonclassical MHC molecule in sharks. Additional analyses, including tissue distribution, also suggest that shark UFA and UGA may be functionally similar to CD1. The discovery of *UFA/UGA/CD1* in Elasmobranchs argues that while it was lost in teleosts and amphibians, it is actually contemporaneous with the birth of classical class I and II and originally located in the primordial MHC.

Examining the presence, distribution, and intracellular signal output of Goldfish leukocyte immune type receptor proteins using a newly developed polyclonal antibody

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Immune cells are pivotal in recognizing and addressing harmful intruders, including bacteria, viruses, fungi, and malfunctioning or injured cells within the body, and can do so using intricate networks of immunoregulatory receptors both on their cell surfaces and intracellularly. Immunoregulatory receptor proteins consist of ligand (e.g., pathogens) binding regions and intracellular signaling currencies that translate these ligand signals into chemical messages, determining whether a cell activates or suppresses the immune response. While extensive research has expanded our understanding of immune cells and immunoreceptors in humans and other mammals, their counterparts in non-mammalian species such as fish are relatively unexplored.

Using Goldfish as a comparative model system, my research aimed to use a recently generated antibody against Goldfish leukocyte immune-type receptor 3 (LITR 3) to assess the presence and distribution of this LITR-type on goldfish kidney immune cell populations using fluorescent microscopy. LITRs represent a group of immunoregulatory receptors found in teleost fishes that bear a resemblance to multiple families of mammalian immunoregulatory receptors. My initial research has shown that the polyclonal antibody reactive to LITR 3 stains Goldfish kidney neutrophils and macrophages, which are the main players in innate immunity. Again, using the polyclonal antibody generated, I was able to identify and tease out the activation of the Mitogen-Activated Protein Kinase pathway, which is known to regulate cell differentiation, proliferation, and apoptosis. This research will seek to improve our understanding of the distribution and function of LITRs in Goldfish tissues, and their importance as an immunoregulatory protein during the induction and control of various antimicrobial responses.

Secretory IgM (sIgM) is an ancient master regulator of microbiota homeostasis and metabolism

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The co-evolutionary relationship between secretory immunoglobulins (sIgs) and microbiota dates back over half a billion years. Coating of gut microbiota by secretory immunoglobulins (sIgs) determines which bacteria colonizes the gut while also it directly influences bacterial metabolism. Our current understanding of this mechanism of homeostatic and metabolic control of microbes is essentially limited to sIgA in endotherms and sIgT in ectotherms. Yet, recent studies have shown that in addition to sIgA and sIgT, sIgM also coats a substantial proportion of the human and fish gut microbiota. This suggests an important and conserved role for sIgM in the maintenance of microbiome homeostasis while it also challenges the dogma that sIgA and sIgT are the key players in that role. To investigate this hypothesis, we conducted temporary and selective depletion of IgM in rainbow trout (*Oncorhynchus mykiss*), a bony fish species. Our findings show that IgM depletion led to a significant loss of gut-associated bacteria. This was accompanied by bacterial translocation into the gut tissue, as well as severe gut tissue damage and inflammation. Critically, IgM-depletion led to long-lasting shifts in the gut microbial community composition, thus indicating a role for sIgM in the control of microbiome homeostasis. In support of a protective role for sIgM in the gut, high mortalities of IgM-depleted fish occurred in a DSS-induced colitis fish model. Interestingly, significant increases in beneficial bacterial metabolites were detected in the gut of IgM-depleted fish thus pointing to a role for sIgM in the modulation of microbial metabolism. Our findings uncover sIgM as an ancient master regulator of microbiota homeostasis and metabolism.

Is IgW the shark mucosal Ig?

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Elasmobranchs have three major immunoglobulin (Ig) isotypes, IgM, IgNAR, and IgW. IgNAR expression is limited to spleen and epigonal, and it has been best been studied for its role in systemic adaptive immunity. IgM is broadly expressed, its monomeric (7S) form clearly involved in adaptive immunity with much less known concerning its pentameric (19S) form. IgW (IgNARC, IgR) has been poorly studied beyond molecular analyses and one biochemical study. Two previous reports, in nurse shark and banded houndshark, demonstrated that IgW is expressed in spleen and epigonal like IgNAR and IgM, but also enriched for expression in the pancreas. Rabbit antisera prepared to two IgW variable domains were generated, both of which immunoprecipitated IgW of the predicted molecular weight from metabolically labeled pancreatic lymphocytes and spleen. FACS studies of three sharks demonstrated high levels of IgW-positive lymphocytes isolated from pancreas in two animals with apparently very few IgM- and IgNAR-expressing B cells. Monoclonal antibodies generated to the recombinant form of IgW also show this unusual expression. We speculate that IgW is secreted into the lumen of the gut via the pancreatic duct serving as the shark mucosal Ig.

The role of a newly discovered toxin in African lungfish aestivation

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Every year, the unfavorable dry season induces the African lungfish to undergo a process known as aestivation. While aestivating, the African lungfish drastically reduces its metabolism and starts producing large amounts of mucus, which eventually harden to form a cocoon with important antimicrobial properties. Among the components of the cocoon, a toxin molecule named AfricCTxA has lately been discovered. The genes encoding for AfricCTxA are expressed by the African lungfish dermal stem cells and upregulated upon aestivation. In Australian lungfish, which does not undergo aestivation, three single exon toxin genes coding for a AfricCTxA variant, AustrCTxA, were found. This fact raises questions about the function of AustrCTxA. The current study explores the biological activity of both recombinant toxins, in relation to the skin remodeling processes underlying African lungfish aestivation. The results obtained thus far suggest a higher toxicity of AustrCTxA compared to AfricCTxA, resulting in higher mortality in zebrafish embryos. SEM revealed that exposure to both recombinant toxins induced skin damage in zebrafish, with AfricCTxA inducing an epidermal rippling phenotype not observed in AustrCTxA treated fish. Both toxins induced neutrophil migration towards the ventral yolk-sac. Ex vivo, exposure of African lungfish skin explants to each toxin showed that AfricCTxA caused epithelial cell apoptosis and epithelial detachment, while AustrCTxA led to a major degradation of the epithelial and muscular tissue. Taken together these outcomes confirm the potential of both toxins to trigger an immune response, with AfricCTxA being particularly associated with decreased toxicity and controlled skin remodeling. Further analyses are ongoing to assess the molecular mechanisms underlying such processes.

Antimicrobial Peptide Genes of the North American Wood Frog (*Rana sylvatica*) and Confirmation of Peptide Expression in Skin

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Antimicrobial peptides (AMPs) are short, amphipathic peptides that exhibit broad-spectrum antimicrobial activity, making them an important element of the immune system of many organisms. The North American wood frog (*Rana sylvatica*) was previously known to produce only two AMPs, brevinin-1SY and temporin-1SY, the latter observed only in recent metamorphs. This is an unusually low number given that many Ranid frogs are known to secrete a wealth of diverse AMPs from their skin. Leveraging the recently released *R. sylvatica* genome assembly, we assembled transcriptomes of *R. sylvatica* skin tissue from existing sequencing read archives. Alongside the two previously identified AMPs, genes encoding eight novel peptides homologous to known Ranid skin-secreted AMPs have been identified, as well as two putative cathelicidins. cDNA sequences matching the predicted AMP transcripts were obtained from the dorsal skin of locally captured *R. sylvatica* via RACE-PCR, and the presence of predicted AMPs was confirmed by tandem ESI-FT mass spectrometry of skin secretions. Additionally, MALDI-TOF mass spectrometry was applied to compare AMP presence across seasonal samples. Lastly, bioinformatic analyses of the genomic regions surrounding the AMP gene loci provide insight into the factors regulating AMP gene expression. Our research represents the first analysis of AMP gene structure in *R. sylvatica*, and the most thorough investigation in any Ranid frog to date, significantly expanding the known AMP repertoire of the species and providing a framework for AMP discovery in related frogs.
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Type VI secretion systems and motility are instrumental for the pathogenicity of *Vibrio diazotrophicus* in purple sea urchin larvae.

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The larval stage of the purple sea urchin (*Strongylocentrotus purpuratus*) live for several months in microbe-rich seawater. Larvae are free-swimming, feeding, and have a well-characterized, complex immune system. In response to exposure to the marine bacterium *Vibrio diazotrophicus,* larvae mount a robust cellular and transcriptional response that includes upregulation of the cytokine interleukin 17 (IL-17) within the gut epithelium and the migration of pigment cells from the ectoderm to the gut. The goal of this study is to identify the mechanisms by which *V. diazotrophicus* elicits this larval immune response. *V. diazotrophicus* isolates were genetically modified using a novel recA-based recombineering system to generate mutants with impaired motility and type VI secretion systems (T6SS). *S. purpuratus* larvae (7 days post fertilization) were exposed to the mutant strains and monitored for cellular responses. Results show that larvae exposed to non-motile *Vibrio* strains have minor reductions in pigment cell activation; in contrast, T6SS mutants elicited only minor inflammatory responses. Bacterial localization was monitored using *in situ* hybridization with 16S rRNA probes and *in vivo* microscopy with fluorescently-labeled *V. diazotrophicus*. IL-17 transcript level was quantified to compare larval responses to generated mutants compared to controls. Together, results indicate that the type VI secretion system plays a major role in pathogenesis while flagellar activity also contributes to a lesser extent. Future studies will define specific effectors involved with the type VI secretion response and what specific function bacterial motility aids in this process.