TLR3 and TLR22; and with R848, which induces IFNs via TLR7. Poly (I:C) strongly induced IFNa in cell lines while the other IFNs showed little response suggesting that IFNa is the main IFN subtype induced through the RIG-I/MDA5 pathway. In contrast, IFNb and IFNc are the main IFNs induced through the TLR7 pathway since R848 induced high transcript levels of IFNb and IFNc and low transcript levels of IFNa in head kidney and spleen. The fact that poly (I:C) induced IFNc strongly in vivo, but little in cell lines, suggests that IFNc is induced through a pathway different from RIG-I/MDA5 or TLR7. IFNd was constitutively expressed in cells and organs, but showed no response to poly (I:C) or R848. Fluorescence in situ hybridization studies showed that poly (I:C) induced IFNa and IFNc in cells lining the sinusoids of head kidney and spleen and in gill pillar cells. Moreover, poly (I:C) strongly induced IFNa throughout the liver tissue while IFNc was little and IFNb not expressed. R848 induced co-expression of IFNb and IFNc in distinct cells in head kidney and spleen. These cells are likely to be specialized high IFN producers as they were few in numbers despite high IFNb/IFNc transcript levels in the same organs. High IFN expression in response to TLR7 ligation is a feature shared by mammalian plasmacytoid dendritic cells.

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O-222.

Making the most of stress: cooperation between skin commensals and the mucosal immune system in rainbow trout

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Abstract
Vertebrate mucosal surfaces are at the interface between environment and their internal milieu where millions of commensal microorganisms live in symbiosis. Thus, commensals receive signals from the environment and from the host. Vertebrates have developed exquisite mechanisms to distinguish between commensals and pathogens. Amongst these mechanisms, mucosal immunoglobulins (Igs) coat commensals in a process known as immune exclusion. However, commensals are not innocuous and if the homeostasis between commensals and vertebrate mucosal immune system is broken, they may translocate across the mucosal epithelium entering the host. Stress is part of the everyday life of animals, including teleost fish. Whether natural or artificial, stress is known to increase disease susceptibility in teleosts. The aim of this study was to investigate whether rainbow trout skin-associated microbiota is able to sense stress and help the host against pathogen invasion. Trout skin tissues and skin-associated bacteria were collected before and after a 5-hour transport stress. Microbiological analysis showed that stress results in significantly higher numbers of culturable bacteria (between 10 and 50-fold) in the skin compared to control fish. The increased number of bacteria was also supported by SEM analysis, which showed high amounts of mucus with associated bacteria in the stressed fish. Selected bacterial strains were grown in the presence of norepinephrine and hydrocortisone and bacterial growth curves were measured. This revealed that stress hormones differentially affect the growth of different trout skin commensals. Moreover, the level of coating of skin bacteria was studied by immunofluorescence using trout anti-IgT and anti-IgM antibodies. We found that stressed trout have 20% more coated bacteria, mostly due to an increase in the double (IgM and IgT) coated population. Furthermore, qPCR analysis of selected immune related genes showed a modification in the expression of Igs and epithelial barrier integrity proteins in the skin. Stress increased both the expression of skin Igs and tight junction genes. These results indicate that trout skin commensals are able to sense and respond differentially to stress and that fish are capable of regulating immune exclusion in order to cope with stress-derived bacterial growth in the skin.

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O-472.

Novel T cell subpopulations expressing CD4-1 and CD4-2 molecules in rainbow trout

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Abstract
In higher vertebrates, the TCR co-receptor CD4 is the key marker of helper T (Th) cells. The CD4 structure consists of four Ig-like domains which are evolutionarily conserved from teleost to mammals. In addition to this classical CD4 molecule (CD4-1 in teleosts), teleost fish possess a second CD4-like gene (CD4-2 or CD4REL), which encodes either two or three Ig-like domains. Importantly, both CD4 molecules have a CXC motif in the cytoplasmic tail, which is a typical CD4 signature for interacting with tyrosine protein kinase p56lck. Recently, antibodies to ginbuna CD4-1 and pufferfish CD4-2 were developed and enable the separation of $CD4^+$ T cells in teleosts. Functional analyses of each antibody-positive cell population suggest the presence of helper $CD4\text{-}1^+$ T cells and $CD4\text{-}2^+CD25\text{-like}^+$ regulatory T cells in ginbuna and Tetraodon, respectively. While flounder CD4-1 and CD4-2 transcripts were detected in different cell populations, it has been largely unknown whether teleost CD4-1 and CD4-2 molecules are co-expressed or distinctively expressed on different cell subsets. Here, to study CD4-1 and CD4-2 expression patterns as well as T cell responses in teleosts, we have developed several monoclonal antibodies (mAbs) against trout CD4-1 and CD4-2. Established mAbs against CD4-1 and CD4-2 could react with cell lines expressing CD4-1 and CD4-2, respectively, but not with untransfected cell lines. Anti-CD4-1 mAbs did not recognize CD4-2 expressing cell lines and vice versa. Peripheral blood leukocytes (PBL), splenocytes and pronephrocytes were stained with anti-CD4-1, anti-CD4-2 and anti-IgM/T or anti-CD8alpha mAbs. Flow cytometric analyses revealed that both CD4-1 and CD4-2 were mainly expressed on lymphocyte populations devoid of CD8alpha or IgM/T expression. Notably, CD4-1/CD4-2 double-positive (DP) cells, CD4-1 single-positive (SP) and CD4-2 SP cells were observed, and among these populations the CD4-1/CD4-2 DP cells represented the predominant population (8-24%) in lymphocyte populations from spleen and pronephros. In addition these organs also contained a significant CD4-2 SP subset. In contrast, CD4-1 SP cells appeared to be the main $CD4^+$ T cell population in PBLs, followed by CD4-1/CD4-2 DP cells, while CD4-2 SP cells were almost negligible. To our knowledge this is the first demonstration of CD4-1 and CD4-2 co-staining in teleosts. Importantly, these new anti-CD4-1 and anti-CD4-2 mAbs can identify SP subsets. Functional studies on antigen-specific proliferation and immunoregulatory functions in these populations are underway.

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O-473.

Identification of regulatory B and T cell subsets in teleost fish

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Abstract
IL-10 is an immunoregulatory cytokine with broad potent anti-inflammatory activities, which is produced by variety of cells, including antigen presentation cells (APCs), T helper 2 cells, regulatory T cells (Tregs), and regulatory B cells (Bregs). A collaborative relationship between Bregs and Tregs has been demonstrated in mammals. Notably, the fact that B cells can display both an antigen-specific B cell receptor and Toll-like receptors suggests that Bregs are able to be involved in immune responses earlier than Tregs and that IL-10 from Bregs as well as APCs is crucial for the generation and/or maintenance of the pool of Tregs. In teleost, IL-10 genes have been identified in some fish species including Cyprinidae and Salmonidae. Interestingly, goldfish IL-10 was revealed to possess the inhibiting bioactivity of inflammatory responses in vitro. However, the cell types producing transcripts and proteins of IL-10 are largely unknown in teleost and the present of Tregs and Bregs remains to be demonstrated. To determine whether teleosts have B cell subset homologous to mammalian Bregs, we sorted IgM^+ and IgT^+ B cells from rainbow trout infected with *Yersinia ruckeri* (*Y. ruckeri*) or *Ceratomyxa shasta* (*C. shasta*) and analyzed the transcription levels of IL-10 in these B cell subpopulations. Moreover, we developed rat monoclonal and rabbit polyclonal antibodies against recombinantly produced rainbow trout IL-10 to detect the presence of IL-10 producing cells. In *Y. ruckeri*-infected fish, only spleen IgT^+ B cells induced statistically significant higher IL-10 transcripts when compared to control fish. Moreover, IgT^+ B cells represented the spleen leukocyte population expressing the highest IL-10 transcript levels. Interestingly, *C. Shasta*-infected fish showed an increase in IL-10 transcripts for both gut IgM^+ and IgT^+ B cell subsets when compared to control fish. Immunohistochemistry studies using anti-rainbow trout IL-10 Abs showed that only a small percentage of the IgM^+ or IgT^+ B cells stained positively for Il-10. Recently, we developed antibodies specific to rainbow trout CD4 molecules. Whether these $CD4^+$ cells produce IL-10 is currently under investigation. Thus far our results show for the first time the presence of IL-10 producing B cells in fish or any other non-mammalian species.

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O-337.

Requirement of contact for direct antibacterial activity of lymphocyte subpopulations in ginbuna crucian carp

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Abstract
Objective: Cytotoxic T-lymphocytes (CTL) recognize and kill cells infected with viruses and intracellular bacteria and tumors in MHC-restricted and antigen-specific manner. In addition to these activities, recent studies in mammals have suggested that CTL can exhibit direct microbicidal activity. In our previous study we found the direct antibacterial activity of $CD4^+$ T-cells and $sIgM^+$ cells as well as $CD8\alpha^+$ T-cells in ginbuna. However, the requirement of contact has yet to be investigated.
Methods: Clonal ginbuna crucian carp *Carrasius auratus langsdorfii* weighing 20-35g were immunized with formalin-inactivated *Edwardsiella tarda. Streptococcus iniae* and *Echerichia coli* were also used as targets. Sensitized and non-sensitized effector lymphocytes ($CD8\alpha^+$, $CD4^+$ and $sIgM^+$) separated by MACS were incubated with target bacteria with E:T ratio of 10^3:1 for 4 hours at 26° C followed by CFU assay. In order to investigate the requirement of contact between effectors and targets, killing activity of supernatants from effector lymphocytes co-cultured with bacteria was tested. Effects of the presence of membrane as a separator between effectors and targets using tissue culture insert were also examined.
Results: $CD8\alpha^+$ T-cells from *E. tarda* immunized ginbuna killed 90%, 100% and 72% of *E. tarda*, *S. iniae* and *E. coli*, respectively. $CD8\alpha^+$ T-cells from non-immunized fish showed similar but slightly lower killing activity than sensitized cells. $CD4^+$ and $sIgM^+$ also showed high killing activity against *E. tarda* and *S. iniae* as found for $CD8\alpha^+$ T-cells, although the activity was lower against *E. coli*. Supernatants from all three types of lymphocytes showed microbicidal activity, although the activity was lower than that evoked by effector lymphocytes. Furthermore, presence of a membrane between effectors and targets did not affect the killing activity.
Discussion: In the present study both sensitized and non-sensitized lymphocytes non- specifically killed target bacteria without the need of contact. However, results obtained in the present study is different from those in previous study with E:T ratio of 10:1 and sensitization of effector donor is required and non-sensitized effector cells exhibited quite low killing activity (around 15%). Furthermore, sensitized lymphocytes more effectively killed bacteria used for immnogen than third-party bacteria suggesting that killing activity is partly specific. Major difference between two experiments is the E:T ratio and effector cells may secret smaller amounts of killing substance in the present study. We suspect that there are two different mechanisms in the direct microbicidal killing by lymphocytes in ginbuna.

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O-423.

Mucosal T-cells in rainbow trout (*Oncorhynchus mykiss*)

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Abstract
T-Lymphocytes act as the cellular effectors in the adaptive immune system in vertebrates. They evolve and mature in thymus and display regulatory as well as killing activity after recognition of foreign antigens presented via MHC I or MHC II, respectively. Two main populations can be divided by their distribution pattern and expression of T-cell receptor heterodimers: α/β T-cells in periphery and γ/δ T-cells in mucosal organs. In bony fish α/β as well as γ/δ T-cells can be detected by the mRNA expression pattern. However, it was not possible yet to investigate their individual tissue distribution or involvement in different immune functions.
Here we described the characteristic of a new monoclonal antibody (mab 89) recognizing a surface marker specifically expressed on T-cells from in mucosal organs (gut, gills, skin) in trout. This marker is restricted to about 10% of thymocytes, not expressed on peripheral T-cells but on all mucosal T-cells. Other leukocyte populations like B-cells or thrombocytes also do not express this marker. In mucosal T-cells immunomagnetically enriched using this new mab equal amounts of TCR α and γ were measured indicating the existence of both α/β and γ/δ T-cells subsets in mucosal organs of trout. By double staining with anti-trout CD8 mabs mucosal T-cells could be divided into CD8+ and CD8- T-cells. Interestingly, a CD8+ leukocyte subset not expressing the marker recognized by mab 89 was detected. Whereas in CD8+ mucosal T-cells CD8β transcripts are expressed at much lower level than CD8α transcripts, the expression level between CD4-1 and CD4-2 transcripts did not differ significantly.
The usage of this new established mab allows, in combination with an earlier described panel of lineage specific mabs (anti trout-IgM, -CD8; -thrombocytes; -myeloid cells; -peripheral T-cells), the detection and isolation of peripheral as well as mucosal leukocyte subsets in rainbow trout to characterize their functions in immune response in detail.

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