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平成　２８　年　　６　月　　７　日現在

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研究課題名（和文）魚類免疫機構における重要なサイトカインIL-2/15/15Lファミリーの解明

研究課題名（英文）Elucidation of the important IL-2/15 /15L cytokine family functions in the fish immune system

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　4,100,000円

研究成果の概要（和文）：マスのIL-2，-15，-15Lが、魚類・哺乳類のグループの枠を超え、哺乳類IL-15Raと交叉反応する事を見出した。また逆に哺乳類のそれらのサイトカインもIL-2を除き、グループの枠を超え魚類IL-15Raと交叉反応する事を見出した。哺乳類IL-2は、哺乳類IL-2Raとのみ反応した。またニジマスのIL-2Rb，IL-21Ra またはIL-2gのエクトドメインを対応するウシ受容体鎖の細胞膜及び細胞質ドメインと結合させたものを同時にトランスフェクションした細胞で、キメラ受容体を介して細胞シグナルを付与できることを明らかにした。真骨魚類で真のIL-5ファミリー遺伝子を初めて発見した。

研究成果の概要（英文）：For IL-2/15/15L cytokines we found that the trout cytokines IL-2, IL-15, and IL-15L cross-react with IL-15Ra across fish-mammal borders, and vice versa, except for mammalian IL-2 which only binds mammalian IL-2Ra. We found that trout cytokines IL-2, IL-15, and IL-15L are N-glycosylated and have functional effects on trout lymphocytes, and that they can signal through chimera receptor chains on co-transfected cells which consist of rainbow trout IL-2Rb, IL-21Ra or IL-2Rg ectodomains that were combined with the transmembrane + cytoplasmic domains of the corresponding bovine receptor chains. We are currently trying to repeat the effect by adding exogenous instead of co-transfected trout cytokines. In addition, we found that recombinant carp IL-2 can help with the establishment of permanent T cell cultures. Furthermore, we were the first to find bona fide genes of the IL-5 cytokine family in teleost fish.

研究分野：　Immunology, Evolution, Fish

キーワード：　Fish　Immunology　Evolution　Cytokine　Interleukin 2　Interleukin 15　Interleukin 15-like　Receptor

１．研究開始当初の背景

The related cytokines interleukin 2 (IL-2) and IL-15 are renowned for their strong power to stimulate lymphocyte proliferation, and play important roles in the mammalian immune system. Their recombinant forms are used and/or tested as medical drugs and vaccine components, while they are also established reagents for laboratory research (e.g. for culturing cells). IL-2 and IL-15 share overlapping but also have different functions. The most characteristic difference is that IL-2 is crucial for the stimulation of regulatory T cells (Tregs) while IL-15 is especially important for the stimulation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. These differences are largely mediated through their cytokine-specific receptor chains, the related IL-2Rα and IL-15Rα chains, respectively. IL-2Rα is involved in binding free IL-2 as part of IL-2Rα· IL-2Rβ· IL-2Rγ receptor complexes, whereas IL-15Rα forms heterodimers with IL-15 that can be presented in free or membrane-bound form to (opposing) IL-2Rβ· IL-2Rγ expressing cells.

Recently, in addition to IL-2 and IL-15 genes, we and others identified a previously unknown but ancient gene “IL-15-like” (IL-15L) in a wide variety of jawed vertebrate species, including many mammals although not human or mouse. All investigated teleost fishes appear to have an intact IL-15L gene. Before the beginning of this project, we had already determined that bovine IL-15L can bind bovine IL-15Rα, but for the fish cytokines the interacting receptor chains were not known. We probably are the only research group worldwide, either in fish or in mammals, which studies IL-15L function.

２．研究の目的

The purpose was to understand the evolution of the IL-2/IL-15/IL-15L cytokine family, particularly in fish, with the hope that this knowledge might lead to use of these cytokines as fish vaccine adjuvants.

３．研究の方法

Gene searches in public databases were based on a combination of gene prediction software programs and our knowledge of where the gene was expected and of family-typical features. Cattle (*Bos bovis*), rainbow trout *(Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) were kept under standard conditions, in agreement with animal welfare rules. RNA, cDNA and sequencing experiments were done by standard protocols, using repeats for verification. For expression of recombinant cytokines and receptor chains, the respective genes were cloned into expression vectors with a strong eukaryotic promoter (the CMV IE promoter) and, in some cases, under addition of a tag sequence. Co-transfection of a human cell line for IL-15Rα or IL-2Rα receptor chains and cytokines allowed monitoring of binding by detection of the cytokine-tag at the cell-surface using specific antibody and fluorescence-activated cell sorting (FACS). Supernatants of transfected human cell line were used as a source of the recombinant cytokine for functional studies. Whether the supernatants could induce STAT3 or STAT5 phosphorylation in peripheral blood mononuclear cells (PBMC) or other leukocyte fractions upon incubation was estimated by anti-pSTAT3 and anti-pSTAT5 Western blot analyses. Whether the supernatants could stimulate trout PBMC was also investigated by RT-PCR analysis for expression of immune marker genes. In order to determine the signaling type I receptor of the fish cytokines, mammalian cell lines were transfected with combinations of IL-21Rγ with either IL-2Rβ or IL-21Rα, followed by incubation with cytokine containing supernatant (see above) and anti-pSTAT3 or anti-pSTAT5 Western blotting. The full-length trout type I receptor chains did not give positive results in these assays, which is why chimera consisting of the corresponding ectodomain part and transmembrane + cytoplasmic domains part of trout and bovine receptor chains were created. In the above described system using supernatants, the chimera receptors gave weak positive results at best, but when the chimera and cytokines were co-transfected to the same cells, STAT phosphorylation was clearly enhanced.

４．研究成果

Our work in the period 2013-2016 included writing and publishing seven studies on the evolution of the immune system, of which two were specifically dedicated to the IL-2/15/15L family [references 3 and 4], one of which was published in Nature [3]. In addition we acquired many data on IL-2/15/15L which we did not publish yet,

because we like to save them for a large publication. Some of those unpublished results are shown here.

We found that the trout cytokines IL-2, IL-15, and IL-15L cross-react with IL-15Rα across fish-mammal borders, and vice versa, except for mammalian IL-2 which only binds mammalian IL-2Rα. We did this by co-transfection experiments followed by FACS analysis for cell surface presence of the tagged cytokine. See Fig. 1.

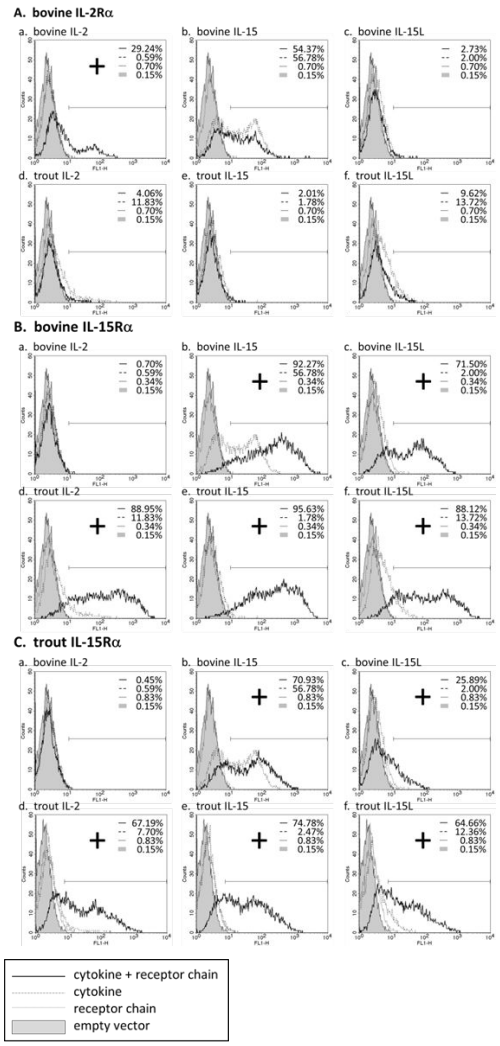

Fig. 1. Presentation of bovine and rainbow trout IL-2, IL-15 and IL-15L by IL-2Rα or IL-15Rα of those species. Live transfected human HEK293 cells were analyzed by FACS using anti-FLAG antibody to detect the presence of the FLAG-tagged cytokines on the cell surface. The amounts of cytokine positive cells were compared between cytokine/receptor co-transfected cells (bold line) and cytokine single transfected cells (dashed line). If the percentage of anti-FLAG-labeled cells increased with co-transfection of receptor chain, it was concluded that the cytokine was bound to receptor chain (shown as "+"). All presented data are from a single representative experiment, but all results were found at least three times.

We found that trout cytokines IL-2, IL-15, and IL-15L are N-glycosylated. See Fig. 2.

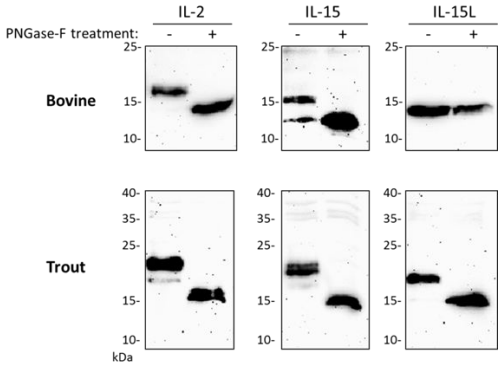

Fig. 2. Bovine IL-2 and IL-15, and rainbow trout IL-2, IL-15 and IL-15L are N-glycosylated. Lysates of transfected cells were digested with PNGase-F (+) or mock treated (-), and analyzed by Western blotting using anti-FLAG. All analyzed molecules except for bovine IL-15L showed a shift in apparent molecular weight consistent with removal of N-linked sugar chains.

We also found that the trout cytokines when incubated with trout leukocytes can induce phosphorylation of STAT5 (see Fig. 3) and expression of several immune genes (see Fig. 4). We have to investigate this better, but it appears that trout IL-15L is more efficient when forming a heterodimer with soluble IL-15Rα (Figs. 3 and 4), and that it can induce a different type of immune response than IL-2 and IL-15 (Fig. 4). For the bovine cytokines we could find STAT activating functions for IL-2, IL-15, and IL-21, but not for IL-15L (data not shown).

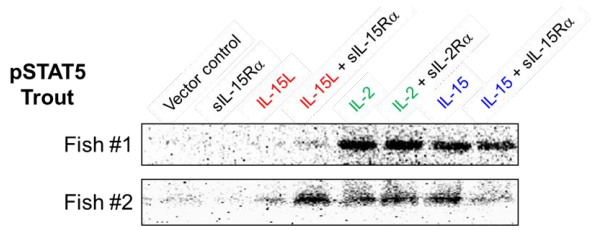

Fig. 3. Phosphorylation of STAT5 in rainbow trout leukocytes induced by recombinant trout cytokines. Trout leukocytes were isolated from spleen and pronephros, and were incubated for 30 min at 15 °C with the supernatants of HEK293 cells transfected for trout IL-2, IL-15 and IL-15L and IL-21, with or without a

soluble form of IL-15Rα (as indicated above the lanes). After incubation, leukocyte lysates were subjected to Western blot analysis using an appropriate antibody to assess the amounts of phosphorylated STAT5 (pSTAT5). The experiments were done using leukocytes of different trout individuals, with the results for two of them (Fish #1 and #2) shown here.

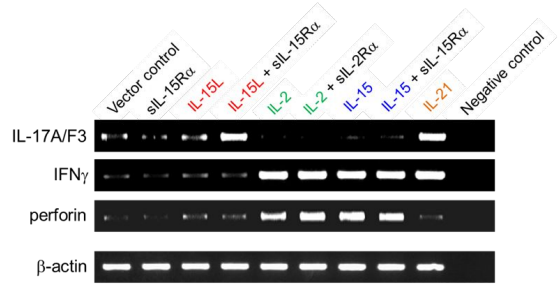

Fig. 4. Gene expression induced by recombinant trout cytokines. Trout leukocytes form pronephros and spleen were incubated for 8 h at 15 °C with the supernatants of HEK293 cells transfected for trout IL-2, IL-15 and IL-15L and IL-21, with or without a soluble form of IL-15Rα (as indicated above the lanes). After incubation, RNA was isolated from the cells and subjected to RT-PCR analysis using specific primers for IL-17A/F3 (a fish gene homologous to mammalian IL-17A and IL-17F and a possible marker for TH17-type immunology), IFNγ and perforin (marker genes for an immune environment dedicated to cell-mediated cytotoxicity) and β-actin (a "house-keeping" control gene).

To determine the type I receptor chains through which IL-15L signals, we made chimeric receptor chains consisting of rainbow trout IL-2Rβ, IL21Rα or IL-2Rγ ectodomains that were combined with the transmembrane + cytoplasmic domains of the corresponding bovine receptor chains. For trout IL-15L, this has allowed us to find induction of STAT5 phosphorylation in a co-transfection experiment with chimera receptor chains (see Fig. 5), and we are currently trying to convincingly replicate this effect by adding exogenous instead of co-transfected trout IL-15L. Although by similar experiments for bovine IL-2, IL-15 and IL-21 we could find the expected functions, we have not been able so far to find a function for bovine IL-15L (data not shown).

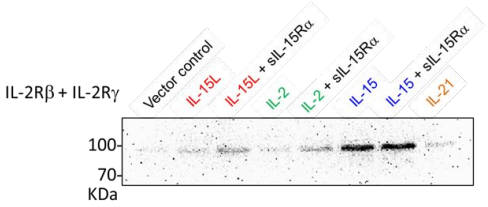

Fig. 5. Phosphorylation of STAT5 in L929 cells induced by co-transfection for various combinations of trout cytokines and trout-bovine chimeric receptor chains. Mouse L929 cells were co-transfected with combinations of expression vectors encoding (1) trout IL-2, IL-15, IL-15L, IL-21 or empty vector, (2) soluble trout IL-15Rα or empty vector, (3) trout-bovine chimeric IL-2Rβ and (4) trout-bovine chimeric IL-2Rγ. Two days after transfection, cells were lysed and assayed for the presence of phosphorylated STAT5 by Western blot analysis.

Additional achievements were:

For carp we got evidence that one of the IL-2 forms, IL-2A, has ability to help with the establishment of permanent T cell cultures, and we are currently investigating whether the IL-2B form has a similar ability (data not shown).

As specialists in fish MHC evolution, we also wrote two comprehensive reviews on fish MHC class I and II genes, which were due because of the recent increase in sequence data across fish species. Both articles were chosen as "editor's pick".

We wrote a review on the evolution of the distinction in immune milieus such as type 1 (characterized by TH1 cells), type 2 (characterized by TH2 cells), type 3 (characterized by TH17 cells), and regulatory (characterized by Treg cells). While doing the investigation for this review, we were the first to find bona fide genes of the IL-5 cytokine family in teleost fish, and we described those in the review [1]. Based on homology searches, the finding of this new teleost cytokine also lead to identification of a yet unknown cytokine in mammals, KK34, about which we recently submitted a paper which we are currently revising based on comments by the reviewers.

５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　6　件）

(1) Takuya Yamaguchi, Fumio Takizawa, Uwe Fischer and Johannes M. Dijkstra (corresponding author). Along the Axis between Type 1 and Type 2 Immunity; Principles Conserved in Evolution from Fish to Mammals. Biology (Basel) Vol. 4, No. 4, 814-859, 2015. doi: 10.3390/biology4040814

(2) Unni Grimholt, Kentaro Tsukamoto, Teruo Azuma, Jong Leong, Ben F. Koop and Johannes M. Dijkstra. A comprehensive analysis of teleost MHC class I sequences. BMC evolutionary biology Vol. 15, 32, 2015. doi: 10.1186/s12862-015-0309-1

(3) Johannes M. Dijkstra. TH2 and Treg candidate genes in elephant shark. Nature Vol. 511, No. 7508, E7-9, 2014. doi: 10.1038/nature13446

(4) Johannes M. Dijkstra (corresponding author), Fumio Takizawa, Uwe Fischer, Maik Friedrich, Veronica Soto-Lampe, Christophe Lefèvre C, Matthias Lenk M, Axel Karger, Taei Matsui and Keiichiro Hashimoto. Identification of a gene for an ancient cytokine, interleukin 15-like, in mammals; interleukins 2 and 15 co-evolved with this third family member, all sharing binding motifs for IL-15Rα. Immunogenetics Vol. 66, No. 2, 93-103, 2014. doi: 10.1007/s00251-013-0747-0

(5) Fumio Takizawa, Kyosuke Araki, Maki Ohtani, Hideaki Toda, Yasutaka Saito, Veronica Soto-Lampe, Johannes M. Dijkstra, Mitsuru Ototake, Tadaaki Moritomo, Teruyuki Nakanishi and Uwe Fischer. Transcription analysis of two Eomesodermin genes in lymphocyte subsets of two teleost species. Fish & shellfish immunology Vol. 36, No. 1, 215-222, 2014. doi: 10.1016/j.fsi.2013.11.004

(6) Johannes M. Dijkstra, Unni Grimholt, Jong Leong, Ben F. Koop and Keiichiro Hashimoto. Comprehensive analysis of MHC class II genes in teleost fish genomes reveals dispensability of the peptide-loading DM system in a large part of vertebrates. BMC evolutionary biology Vol. 13, 260, 2013. doi: 10.1186/1471-2148-13-260

〔学会発表〕(計 10 件)

(1) Johannes M. Dijkstra (invited speaker) TH1, TH2, and other polarizations of immune cells. Fish immunology seminar, at the Laboratory of Fish Pathology, Department of Veterinary Medicine, Nihon University, Fujisawa, Kanagawa, Japan. September 10th, 2015

(2) Takuya Yamaguchi, Uwe Fischer and Johannes M. Dijkstra (speaker). Evolution of the interleukin 2, 15 and 15-like family. The 27th Meeting of the Japanese Association of Developmental & Comparative Immunology, Obama, Japan. August 22nd, 2015

(3) Johannes M. Dijkstra (invited speaker). How to find genes. Technical training & seminar in Fish Disease Lab, at the Laboratory of Fish Pathology, Department of Veterinary Medicine, Nihon University, Fujisawa, Kanagawa, Japan. June 4th, 2015

(4) Johannes M. Dijkstra (poster presentation), Takuya Yamaguchi, Uwe Fsicher, Keiichiro Hashimoto. Review on the (expected) prowess of the cytokine family comprising interleukins 2, -15 and -15-like to initiate an anti-cancer immune status. Keystone Symposia, Tumor Immunology: Multidisciplinary Science Driving Combination Therapy (J7), Banff, Canada. February 9th, 2015

(5) Takuya Yamaguchi and Johannes M. Dijkstra (speaker). Evolution of the interleukin 2, 15 and 15-like family. The 26th Meeting of the Japanese Association of Developmental & Comparative Immunology, Sendai, Japan. July 10th, 2014

(6) Johannes M. Dijkstra. The evolution of the IL-2 cytokine family. Technical training & seminar in Fish Disease Lab, at the Laboratory of Fish Pathology, Department of Veterinary Medicine, Nihon University, Fujisawa, Kanagawa, Japan. February 27th, 2014

(7) Johannes M. Dijkstra (speaker), Fumio Takizawa and Keiichiro Hashimoto. An unexpected, ancient third interleukin -2/15 family member in mammals. The 42nd Annual Meeting of the Japanese Society for Immunology, Chiba, Japan. December 12th, 2013

(8) J.M. Dijkstra (invited speaker). Major Histocompatibility Complex (MHC) in Fish Talk for the Department of Evolutionary Studies of Biosystems, The Graduate University for Advanced Studies (SOKENDAI), Hayama, Kanagawa, Japan.

September 28th, 2013

(9) Johannes M. Dijkstra (speaker), Fumio Takizawa, Uwe Fischer and Keiichiro Hashimoto. The evolution of the three-member IL-2/15/15L cytokine family. The 25th Meeting of the Japanese Association for Developmental & Comparative Immunology, Okayama, Japan. August 27th, 2013

(10) Johannes M. Dijkstra (invited speaker), Unni Grimholt, Jong Leong J, Ben F. Koop and Keiichiro Hashimoto. New inisghts into MHC evolution Fish Immunology seminar, at the Laboratory of Fish Pathology, Department of Veterinary Medicine, Nihon University, Fujisawa, Kanagawa, Japan. June 8th, 2013

〔図書〕（計　0　件）

〔産業財産権〕
○出願状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
出願年月日：
国内外の別：

○取得状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
取得年月日：
国内外の別：

〔その他〕
ホームページ等

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