Fish and Shellfish Immunology 85 (2019) 3–8

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

What could be the mechanisms of immunological memory in fish?

Takuya Yamaguchi[a], Edwige Quillet[c], Pierre Boudinot[b], Uwe Fischer[a,*]

[a] Laboratory of Fish Immunology, Institute of Infectology, Friedrich-Loeffler-Institut, Südufer 10, Greifswald-Insel Riems 17493, Germany
[b] Virologie et Immunologie Moléculaires, INRA, Université Paris-Saclay, 78350 Jouy-en-Josas, France
[c] Génétique animale et biologie intégrative, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

ARTICLE INFO

Keywords:
Rainbow trout
Vaccine
Yersinia ruckeri
Adaptive immunity
Adoptive transfer
Targetfish

ABSTRACT

Vaccination is the most effective strategy to control infectious diseases in species with adaptive immunity. In human and in mouse, vaccination typically induces specific memory cells, which can mediate a fast anamnestic response upon infection by the targeted pathogen. In these species, successful vaccination induces a long-lasting protection, long after the titres of specific antibodies and the frequency of specific T cells have returned to steady state. Vaccination is also an important challenge in aquaculture, since alternative treatments are either too costly, or, in the case of antibiotics, are harmful for the environment or may result in dangerous resistances. However, the mechanisms of the long-term protection elicited by vaccines in fish remain poorly understood. Although fish possess typical B- and T-cells expressing diverse repertoires of immunoglobulins and T-cell receptors, many features of antigen specific responses are different from what is known in mouse and in human. Memory is one of the most elusive properties of fish adaptive immunity, and its basis is widely unknown. In this opinion article, we discuss the concept of immune memory in the context of the fish immunity. We illustrate the complexity of this question by discussing the results of experiments showing that protection can be passed through adoptive transfer of leukocytes from vaccinated donor fish to naive histocompatible recipients. Combined with tools developed in Targetfish and in previous projects, such as monoclonal antibodies against B- and T-cell markers, we propose that such models of protection transfer provide excellent systems to dissect the mechanisms of B- and T-cell memory in the future.

1. How to define immune memory?

Immune memory is a fundamental characteristic of the adaptive immune system in vertebrates. However, the definition of memory is still changing, under the influence of new mechanisms of long-term protection. A recent article [1] put in perspective different points of view about the nature of immunological memory. According to Donna Farber, memory is defined by three conditions: (1) memory immune cells are long-lived and maintained independently of stimulation by the antigen, (2) memory immune cells are epitope specific and (3) memory immune cells are intrinsically changed by the previous encounter with the antigen. Andreas Radbruch and Klaus Rajewsky consider that immunological memory is defined by "a stimulus-specific change of immune reactivity that persists in the absence of the stimulus". Rolf Zinkernagel reminds the classical definition of a memory response as a faster and stronger response against a previously encountered antigen, compared with the primary response, and emphasizes that the key question is whether or not protective immunity is maintained in an antigen-dependent or -independent manner. Finally, Mihail Netea proposes a wider definition of memory as the capacity of the immune system to keep and recall information on encountered antigens or pathogens, which also includes the concept of innate immune memory or trained immunity [2]. While adaptive immune memory involves permanent genetic changes such as mutations and recombinations, innate immune memory is characterized by epigenetic reprogramming involving sustained changes in gene expression and cell physiology. These multiple definitions and concepts underscore the complexity of the question, while these authors essentially refer to the immunity of well-known species of mammals. Overall, however, it is accepted in mammals that vaccination against an infectious pathogen is based on the establishment of populations of memory cells after an intentional immunization with a homologous attenuated/inactivated strain of the corresponding pathogen or with parts of it. Beyond individual protection, mammalian immunological memory is certainly also important for the survival of offsprings *in utero* and during the neonatal life. This is mainly due to antibodies, since protecting antibodies can be transferred from the mother to offspring during the foetal life in many species, and also via colostrum (reviewed in Refs. [3,4]).

* Corresponding author.
E-mail addresses: Takuya.Yamaguchi@fli.de (T. Yamaguchi), Uwe.Fischer@fli.de (U. Fischer).

https://doi.org/10.1016/j.fsi.2018.01.035
Received 26 October 2017; Received in revised form 18 January 2018; Accepted 21 January 2018
Available online 02 February 2018

Importantly, the modalities of secondary responses, which allow assessment of immunological memory, vary considerably across vertebrates. Differences between primary and secondary responses have been observed from fish to mammals. Typical secondary responses, similar to those described in mouse and human, are fast and lead to higher titres of specific antibodies when compared to primary responses; they are found in mammals and birds, where Ig gene hypermutation occurs in germinal centres [5–7]. Early works in frogs proved the existence of secondary responses in "lower" vertebrates, but showed they were not accompanied with a substantial increase in antibody affinity, though Ig genes get hypermutated even in tadpoles [8]. In fish, the modalities of secondary responses and long-term protection are even less similar to the typical situations described in mammals.

2. Secondary response and long-term protection in teleost fish

The existence of a true secondary response with typical characteristics in fish was initially debated [9]: the primary B cell response is generally much slower and develops over several weeks or even months especially in cold water. For example, the antibody response against the hapten dinitrophenyl keyhole limpet haemocyanin (DNP-KLH) in rainbow trout peaked 50 days post immunization, and antibody titres remained high for a long time, at least until 150 days post immunization [10]. Thus, the maintenance of protection might be partly due to a long-term primary response and long-lasting increase of specific antibody titres. However, significant differences between primary and secondary responses were demonstrated in several fish species. A faster secondary response along with elevated antibody titres (about doubled titres) were reported after immunization with bovine serum albumin [11] and with sheep red blood cells (SRBC) [12] in carp, as well as against the pathogenic bacteria *Y. ruckeri* [13] and *Aeromonas salmonicida* [14] in rainbow trout. The dose dependency of the secondary antibody response was studied in detail in carp, further supporting the presence of a cell based immune memory: fish were immunized with 10^5, 10^7 and 10^9 SRBC by intramuscular injection and challenged 1, 3, 6 or 10 months later with the same dose; with 10^9 SRBC, a secondary response was already observed at 3 months and was maintained until later time points, and even with the lowest dose, titres reached comparable or higher levels. However, the high-dose, 10^9 SRBC, did not lead to a clear secondary response with enhanced titres, while a high primary response was induced [12]. These data showed that the secondary response was at least quantitatively higher than the primary response, supporting the existence of a certain level of immunological memory.

The existence of efficient "memory" in fish is also advocated by the (relatively) long-term protection afforded by vaccination. For example, in rainbow trout, bath vaccination with a bacterin of *Y. ruckeri*, the causative agent of enteric red mouth disease (ERM), was shown to provide a significant protection of vaccinated fish 4 and 8 weeks post-vaccination [15]. DNA vaccination of rainbow trout against infectious hematopoietic necrosis (IHN) has been shown to confer protection over a period of two years, which would cover the life cycle of a commercial fish [16]. It remains unclear whether a continuous stimulation of the immune system through long-term expression of the viral G protein expression from the plasmid is required for this protection. Importantly, reporter protein expression from injected plasmids can still be detected over long periods [17,18]. Another example is DNA vaccination against viral haemorrhagic septicaemia (VHS), which was also shown to induce long-lasting protection post vaccination [19]. While protection established shortly after vaccination was cross-protective in terms of a heterologous viral pathogen this cross-protection was absent two months after vaccination, while the specific response to the homologous virus persisted. In most cases, this protection seems to be based on anamnestic secondary responses, which provide a better defence against the pathogens compared to a naive immune system. Analysis of gene expression showed that such a protection was somehow associated with up- or down-regulation events of the expression of immune related molecules such as cytokines and transcription factors [15,20,21]. However, the detailed cellular basis of protection including T- and B-cell dynamics after fish vaccination is often poorly understood and, most importantly, the persistence of the antigen between the initial immunization and the challenge is most often not directly evaluated.

Other key features associated with immune B cell memory formation in mammals are class switch and affinity maturation. While an Ig class switch seems to be absent in teleosts, at least in all species studied in sufficient detail, it is generally believed that most lower vertebrates can hypermutate Ig genes but lack the microenvironment required for the selection of B cells with hypermutated Ig receptors of high affinity for the antigen [22,23]. The enzymatic machinery needed for somatic hypermutation of Ig genes is present in teleosts [24–26], and indeed hypermutation was observed in zebrafish mature Ig repertoires [27]. Interestingly, long-term studies of the antibody responses in rainbow trout showed that high affinity antibodies appear late and become dominant, more than 25 weeks after (primary) immunization [28,29]. The kinetics of antibody affinity in trout immunized with TNP-KLH revealed that a relatively low affinity antibody subpopulation appears early, does not achieve high titres and is transient; an intermediate affinity subpopulation appears later (five weeks after immunization), achieves relatively high titres and persists longer than the low affinity antibodies; and the highest affinity subpopulation emerges only after 15 weeks at still high titres [30,31]. These studies suggest, that a slow affinity maturation may actually occur in fish through alternative mechanisms.

The vast diversity of fish (and their immune systems) should also be considered in investigations about putative immune memory in this group. Thus, species related to cod (Gadidae) and pipefish lack MHC class II and CD4 [32,33], which implies that B cell responses cannot rely on a T cell help as in mammals. In these species, B cell memory, if it exists, must be based on particular pathways and this assumption should be taken into account with regard to the successful vaccination protocols in these fish species.

In this general context, the definition and the putative location of fish memory cells also remain elusive. B cells from rainbow trout head kidney still produced specific antibodies later than 20 weeks post immunization with TNP-KLH [34]. In contrast, spleen or blood leukocytes contained very few or no TNP-specific antibody secreting cells 10–15 weeks after immunization. Thus, rainbow trout anterior kidney antibody secreting cells may resemble mammalian long-lived plasma cells, and they are likely responsible for sustained serum antibody titres [34]. In the channel catfish, it has been proposed that hypermutation of Ig genes may occur in melanomacrophage centres of the spleen, since activation-induced cytidine deaminase (an enzyme causing somatic hypermutation, gene conversion, and class-switch recombination of immunoglobulin genes in mammalian B cells) is expressed in these structures [35], but the cellular context remains to be further dissected in fish (discussed in Ref. [26]). Overall, fish memory cells are very poorly defined and their existence remains debatable.

3. A system to further define the basis of immune memory: transfer of protection through cell transplant in rainbow trout

If fish possess memory cells, their phenotypes, frequency, lifespan and functions remain essentially unknown. The lack of antibodies against key surface markers has certainly hampered progress in this field, making difficult the definition and isolation of relevant cell subsets. Furthermore, the lack of syngeneic or congenic animals has hindered cell transfer experiments, which would help defining a "memory" cell compartment. Taking advantage of the availability of clonal, isogenic rainbow trout, we could recently transfer protection by transplantation of peripheral blood leukocytes (PBL) from vaccinated fish to histocompatible individuals. The potential interest of this experimental set up for the study of immunological memory will be discussed below.

Table 1
Summary of the experimental design, including groups, recorded mortalities and numbers of sampled fish.

Group	Donor	Total No. of fish	Infection status	No. of dead fish	Mortality	No. of FCM-analysed fish		No. of ELISA- analysed fish
						21 dpi	28 dpi	28 dpi
1	non-vaccinated	22	0 (mock)	0	0%	3	3	3
2		22	Low	0	0%	6	6	6
3		22	High	14	63.6%	4	4	4
4	AquavacERM vaccinated	22	0 (mock)	0	0%	3	3	3
5		22	Low	0	0%	6	6	6
6		22	High	0	0%	6	6	6

Isogenic fish were vaccinated by immersion using a commercial vaccine against ERM, and boosted ten weeks later, also by immersion (material and methods in supplements). Control fish of the same isogenic progeny were mock-vaccinated and -boosted. Two weeks after the boost, PBL were transferred to histocompatible, naive recipients from the same fish clone, *i.e.* with the same genetic background. Recipients were challenged by bath with different dosages (mock, low and high) of *Y. ruckeri* twenty-four hours later and mortalities were recorded (Table 1). The high dose challenge led to high mortality (63%) of recipient fish that had received PBL from non-vaccinated donors (group3). While mortalities were observed between day 5 and 12 after challenge, most fish died until day 9. In contrast, fish which had received PBL from vaccinated donors (group 6), were fully protected, indicating that protection against *Y. ruckeri* was conferred to the recipients by adoptive transfer of PBL from vaccinated donors.

Following PBL transfer, dynamics of $CD8\alpha^+$, IgM^+ and IgT^+ lymphocytes in the blood, gills and spleens of recipients that had survived the infection were analysed. We are aware that bath challenge did not ensure that all fish had received the same amount of bacteria, and that the survival might have been affected by this factor; however, these observations allow a first comparison between immune cell populations in protected fish and in the other groups on 22 (Fig. 1a) and 29 days (Fig. 1b; Fig 1c) after transfer, which are corresponding to 21 and 28 days post challenge, respectively.

Twenty two days post transfer, the total number of $CD8\alpha^+$ T cells in non-challenged recipients which had received PBL from non-vaccinated fish (group 1), were about 2×10^4 in PBL and approx. ten times more in spleen and gills (Fig. 1a). In comparison, non-challenged recipients, which were injected with PBL from vaccinated donors (group 4), had much more $CD8\alpha^+$ T cells in the gills (about 2.5 times more than those of group 1), while this trend was not observed in spleen and PBL. This suggests that T cells might have accumulated (or proliferated) in gills three weeks after the transfer. In challenged recipients, twenty-one days after bath infection, the number of $CD8\alpha^+$ T cells was higher in PBL of recipients, which had received cells from non-vaccinated donors (group 2 and 3), but not in those transferred with cells from vaccinated donors (group 5 and 6). This trend was also observed in the spleen of recipients challenged at low dose (compare group 2 to group 5), but not in other cases. This suggests that although recipient groups challenged with low dose did not show mortality, different responses and recruitments of T cells occurred between fish transferred with cells from vaccinated and control fish. These patterns were not observed 28 days post challenge, the most obvious change being then an increase (approx. 3.5 times) of $CD8\alpha^+$ T cells in the spleen of high dose challenged recipient fish, which had received PBL from vaccinated donors (and were protected, group 6). In the same recipient group, there was an increase (approx. 2.5 times) of $CD8\alpha^+$ splenocytes between day 21 and 28 (Fig. 1c) post challenge, while such an increase was not observed in recipient fish which had received PBL from control donors (group 3, in which high mortalities were recorded). To what extent this protection relates to a $CD8\alpha^+$ T cell response remains to be analysed. Of note, no differences in $CD8\alpha^+$ T cell numbers were observed 28 days post challenge, between non-challenged fish, which had received PBL from vaccinated (group 4) and control donors (group 1). Additional studies will be necessary to establish firmly that this kinetics is reproducible and to further elucidate the role of $CD8\alpha^+$ T cells in the transferred protection to ERM.

Similar to $CD8\alpha^+$T cells, twenty two days post transfer, IgM^+ and IgT^+ B cell numbers were about three times increased in gills of non-challenged recipients, which had received PBL from vaccinated donors (group 4) compared to those which had received PBL from non-vaccinated control donors (group 1). Again, this pattern was not found twenty nine days post transfer. The only remarkable trend regarding B cells was observed at the second time point of sampling. 28 days after high dose infection an accumulation (or proliferation) of both IgM^+ and IgT^+ B cells in the spleen of challenged fish was observed, especially in recipients which had received PBL from vaccinated donors (group 6). Combining the most obvious increase in B cell numbers in high dose challenged recipients that have received immune cells from vaccinated donors with the fact they were fully protected (Table 1) points on a role of B cells in the immune response against ERM.

An interesting observation was made in the spleen, where the numbers of $CD8\alpha^+$, IgM^+ and IgT^+ cells of high dose challenged recipient fish, which had received PBL from vaccinated donors (group 6) at 28 d p.i. were higher than in those at 21 d p.i. Such an increase was not observed in low dose challenged (group 1 and 4) or mock challenged (group 2 and 5) recipients (Fig. 1c). This suggests that B- and T-cell responses might continue developing at this stage in the spleen of group 6 recipients at 28 d p.i.

Importantly, such trends should be taken with great caution, since they come from a pool of 3–6 survivors from a single campaign of experiments. Although a single individual with a remarkable phenotype may induce a bias, we believe the strongest trends are interesting. Although the modest numbers of fish used in this first set of experiment do not fully demonstrate trends, they provide a first frame of analysis to be confirmed by further studies. The adoptive transfer model appeared to be excellent to pursue the characterization of mechanisms involved in the transfer of “memory” and/or protection.

In order to check the titres of secreted IgM antibodies against *Y. ruckeri*, sera as well as muci from gills and skin were collected from surviving recipients 28 days after challenge and were analysed by ELISA. Among all examined groups, the titres of anti-*Y. ruckeri* IgM in muci and sera were clearly elevated only in recipients of group 6 that had received PBL from vaccinated donors and that thereafter had been challenged with high dose of *Y. ruckeri* (Fig. 2) further suggesting that a secondary response is dose dependent. Nevertheless, these results indicate that transfer of PBL from vaccinated donors successfully transferred the capacity to produce high titres of anti-*Y. ruckeri* antibodies upon infection in the recipients, hence to mount a “memory” response. This was correlated with high levels of protection. In contrast, recipient fish that had received PBL from non-immunized donors (group 3) showed high mortality after high dose challenge, and survivors did not develop anti-*Y. ruckeri* IgM antibodies. Interestingly, not all recipients of group 6 were seroconverted although full protection and high levels of IgM^+ and IgT^+ cells were recorded. Group 6 can be divided into non- and high IgM responders (which is partially also true for vaccinated

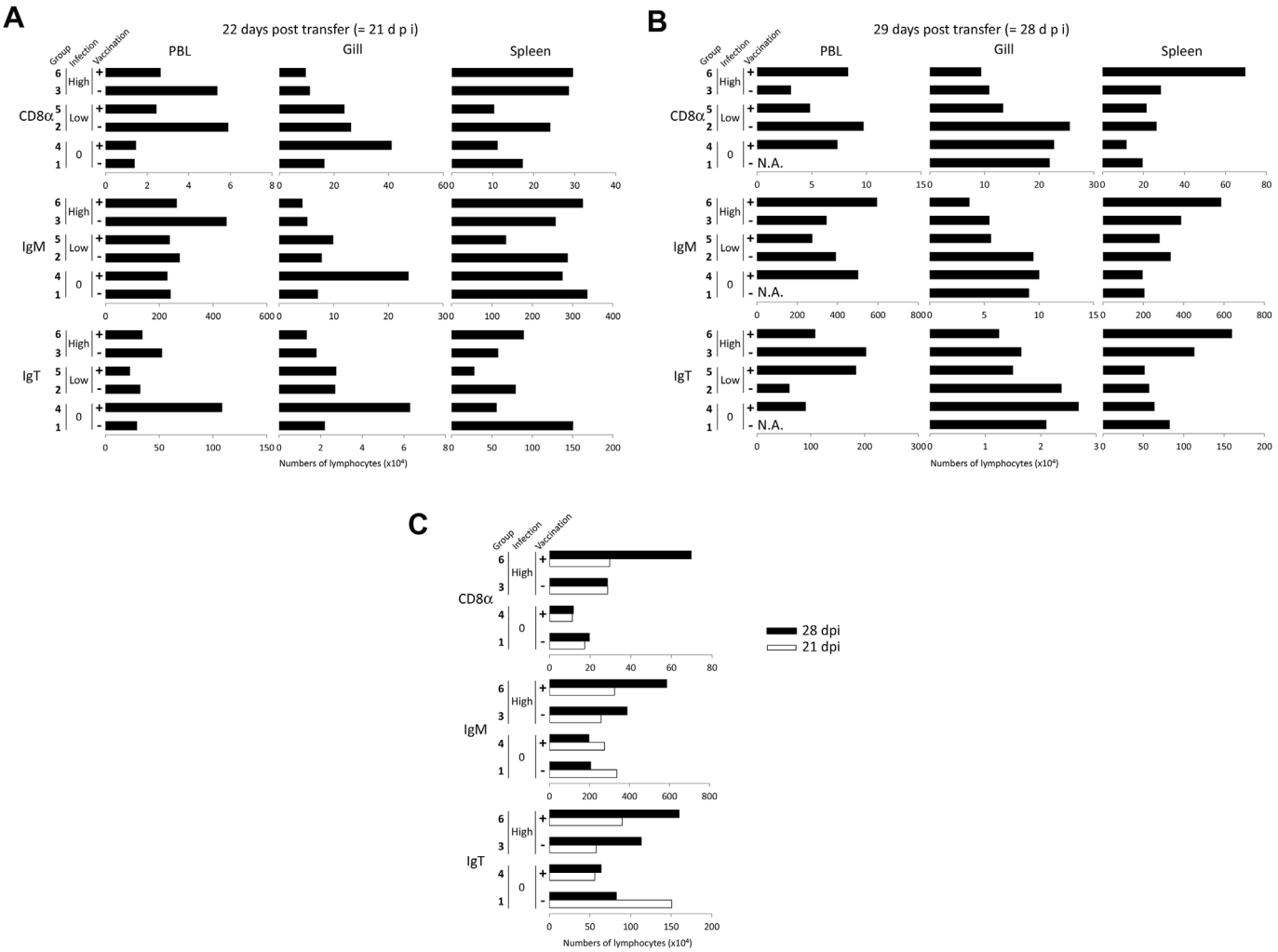

Fig. 1. Numbers of CD8α$^+$, IgM$^+$ and IgT$^+$ leukocytes in six recipient groups. 21 (A) and 28 (B) days after bath (or mock) challenge, percentages of three leukocyte subpopulations in PBL, gill leukocytes and splenocytes were analysed by flow cytometry, and the total numbers of leukocyte subpopulations per fish were calculated. Pooled samples from each group (three to six fish/group; shown in Table 1) were analysed. Data from PBL of group 1 are not available for 28 d p.i. (N.A.) due to a technical failure (blood clotting). In these figures, the results are shown as follows; left vertical panels depict PBL, centre panels gill and right panels spleen; upper horizontal panels depict CD8α$^+$, middle panels IgM$^+$ and lower panels IgT$^+$ cells. (C) Here percentages of the three leukocyte subpopulations in splenocytes from group 1, 3, 4 and 6 were compared on 21 and 28 d p.i. (note the increase of CD8α$^+$ cells in group 6 at day 28). Black bars show the data from day 28 p.i., white bars show the data from 21 d p.i., respectively. The X-axis corresponds to the total numbers of the respective leukocyte subpopulations per fish ($\times$ 10^4).

donors). This implies that several mechanisms may explain survival and that, although genetically identical fish were used in these experiments, immune responses to a given pathogen may show considerable inter-individual variability in fish. These observations underline the importance of following these parameters when tracking immune memory.

4. Perspectives and discussion

The available literature illustrates and the case study described above exemplifies that much remains to be understood and defined regarding the protection against pathogens - including the protection induced by vaccination - and a putative immunological memory in fish. An important issue would be to identify the cellular compartment responsible for the long-term protection. Whether the mechanisms triggered by such cells may be named “memory” would be the next question. Several mechanisms can be proposed on how protection has been transferred from vaccinated donor fish to recipient fish. One possibility is that effector cells were transferred in an activated stage induced by vaccination plus boost, and that thus have directly contributed to rescue recipients from infection. Such activated effector cells could have acted specifically for example via specific antibody production, neutralising the pathogen. It is also reasonable to consider that transferred donor effector cells mounted a more efficient immune response in concert with the hitherto naïve immune system of the recipient. Thus, innate immunity of the recipients complemented by the action of transferred cells could be efficient enough to promote survival. Last but not least, bona fide memory cells could have been transferred to recipients allowing a fast and effective response to the infection and leading to high survival. These different hypotheses could be tested by performing transfer of particular sorted cell subsets, and detailed characterization of the response to different challenges (homologous versus heterologous pathogens, multiples routes). Using a similar experimental set-up in isogenic ginbuna crucian carp [36] where Yamazaki et al. have shown that adoptive transfer of CD4$^+$ and CD8α$^+$ cells from vaccinated donors protects recipients against a homologous *Edwardsiella tarda* challenge. The carriers of protection were CD8α$^+$ cells and, to a lesser extent, CD4$^+$ cells. However in this study, ginbuna lymphocytes were isolated from donor fish only 8 days after immunization, which is probably enough to mount a specific immune responses in this species, but clearly too short to study long-term, persistent “memory” cells.

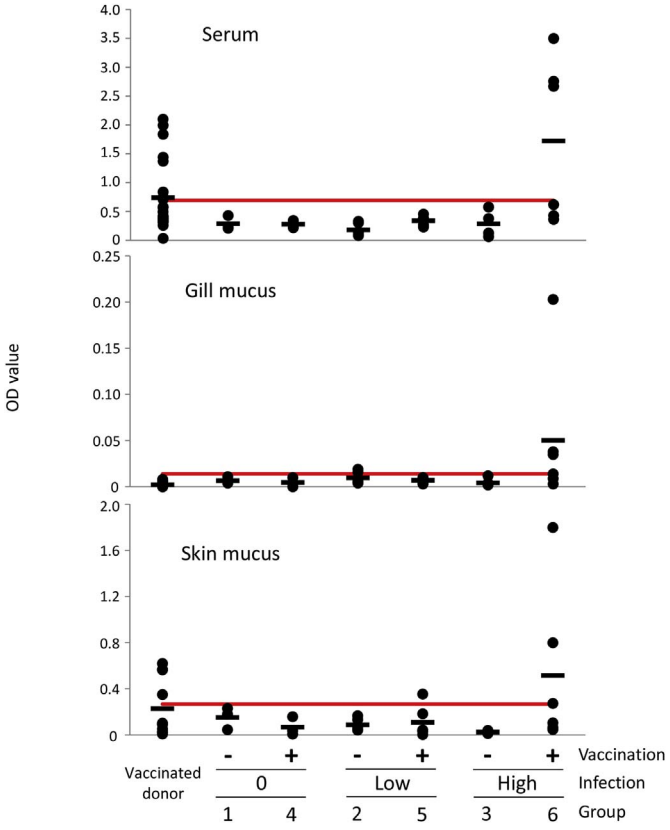

Fig. 2. Titres of anti-*Y. ruckeri* IgM antibodies in the vaccinated donor fish (left dot column) and in the six recipient groups 1 to 6. 28 days after challenge, muci from gill and skin as well as sera were collected from survived fish (three to six fish per group as shown in Table 1) and were individually analysed by ELISA except skin- and gill-muci from vaccinated donor fish. For skin- and gill-muci from vaccinated donor fish, samples from 2 fish were pooled. The Y-axis shows OD values. Dots (●) represent individual OD values. Hyphens (-) represent mean OD values of each group. To distinguish between positive and negative samples, cut-off levels were calculated (threefold standard deviations of negative control ODs plus their corresponding mean OD values) and are shown as red lines.

To get more insights in the mechanisms of long-term protection and memory, transfer experiments of sorted cell subsets should be performed in models similar to the one described above in trout. When the size of the fish and the time line allows, it would be very powerful tool to follow immune parameters such as antigen specific antibody titres in individual fish, using non-lethal sampling, before and after challenge.

In conclusion, models such as the cell transfer between histocompatible fish presented in this article will be instrumental to understand the mechanisms and impact of anamnestic memory responses in fish. These future analyses will benefit from antibodies specific for lymphocytes markers. A better characterization of memory cell markers such as CD27, CD38, CD45RO, CD45RA and CD62L (and production of the corresponding reagents) in fish would be also extremely useful. Besides, the development of new high-throughput transcriptomic approaches will likely be instrumental for the definition of memory cell subsets in fish. Modifications of lymphocyte populations in lymphoid organs after PBL transfer and or challenge suggests that transferred cells might be prone to recruitment into defined territories, or to re-localisation of immune cells.

Acknowledgements

We are grateful to Prof. Oriol Sunyer, University of Pennsylvania, USA, for providing us the monoclonal antibody against IgT, to Prof. Inger Dalsgaard, Danish Technical University for providing us with the Yersinia ruckeri O1 strain and to Prof. Kurt Buchmann, University of Copenhagen, Denmark for technical advice regarding ELISA. We also thank Mrs. Susann Schares for excellent technical assistance. This work was supported by the EU FP7 grant 311993 (TargetFish) for Pierre Boudinot, Takuya Yamaguchi and Uwe Fischer.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2018.01.035.

References

[1] D.L. Farber, M.G. Netea, A. Radbruch, K. Rajewsky, R.M. Zinkernagel, Immunological memory: lessons from the past and a look to the future, Nat. Rev. Immunol. 16 (2016) 124–128 https://doi.org/10.1038/nri.2016.13.
[2] M.G. Netea, L.A. Joosten, E. Latz, K.H. Mills, G. Natoli, H.G. Stunnenberg, L.A. O'Neill, R.J. Xavier, Trained immunity: a program of innate immune memory in health and disease, Science 352 (2016) 1–9 aaf1098 https://doi.org/10.1126/science.aaf1098.
[3] A. Marchant, M. Sadarangani, M. Garand, N. Dauby, V. Verhasselt, L. Pereira, G. Bjornson, C.E. Jones, S.A. Halperin, K.M. Edwards, et al., Maternal immunisation: collaborating with mother nature, Lancet Infect. Dis. 17 (2017) e197–e208 https://doi.org/10.1016/S1473-3099(17)30229-3.
[4] M.F. Jennewein, B. Abu-Raya, Y. Jiang, G. Alter, A. Marchant, Transfer of maternal immunity and programming of the newborn immune system, Semin. Immunopathol. 39 (2017) 605–613 https://doi.org/10.1007/s00281-017-0653-x.
[5] L. Du Pasquier, Meeting the demand for innate and adaptive immunities during evolution, Scand. J. Immunol. 62 (2005) 39–48 https://doi.org/10.1111/j.1365-3083.2005.01608.x.
[6] M. Flajnik, L. du Pasquier, W.E. Paul (Ed.), Evolution of the Immune System in Fundamental Immunology 7th Edn, Lippincott-Williams & Wilkins, Philadelphia, 2012, pp. 67–128.
[7] R. Mehr, H. Edelman, D. Sehgal, R. Mage, Analysis of mutational lineage trees from sites of primary and secondary Ig gene diversification in rabbits and chickens, J. Immunol. 172 (2004) 4790–4796 https://doi.org/10.4049/jimmunol.172.8.4790.
[8] M. Wilson, A. Marcuz, L. du Pasquier, Somatic mutations during an immune response in *Xenopus* tadpoles, Dev. Immunol. 4 (1995) 227–234 https://doi.org/10.1155/1995/38639.
[9] F. Rothe, H. Ambrosius, Contributions on the immunobiology of poikilothermic vertebrates. V. The proliferation of antibody-producing cells in tortoises, Acta Biol. Med. Ger. 21 (1968) 525–536.
[10] M. Cossarini-Dunier, F.X. Desvaux, M. Dorson, Variability in humoral responses to DNP-KLH of rainbow trout (*Salmo gairdneri*). Comparison of antibody kinetics and immunoglobulins spectrotypes between normal trouts and trouts obtained by gynogenesis or self-fertilization, Dev. Comp. Immunol. 10 (1986) 207–217 https://doi.org/10.1016/0145-305X(86)90004-2.
[11] R.R. Avtalion, Temperature effect on antibody production and immunological memory, in carp (*Cyprinus carpio*) immunized against bovine serum albumin (BSA), Immunology 17 (1969) 927–931.
[12] G.T. Rijkers, E.M.H. Frederix-Wolters, W.B. Van Muiswinkel, The immune system of cyprinid fish. The effect of antigen dose and route of administration on the development of immunological memory in carp (*Cyprinus carpio*), in: M.J. Manning (Ed.), Phylogeny of Immunological Memory, Elsevier/North-Holland, Amsterdam, 1980, pp. 93–102.
[13] M. Cossarini-Dunier, Secondary response of rainbow trout (*Salmo gairdneri* Richardson) to DNP-haemocyanin and Yersinia ruckeri, Aquaculture 52 (1986) 81–86 https://doi.org/10.1016/0044-8486(86)90027-X.
[14] M.F. Tatner, A. Adams, W. Leschen, An analysis of the primary and secondary antibody response in intact and thymectomized rainbow trout, Salmo gairdneri richardson, to human gamma globulin and *Aeromonas salmonicida*, J. Fish. Biol. 31 (1987) 177–195 https://doi.org/10.1111/j.1095-8649.1987.tb05224.x.
[15] M.K. Raida, K. Buchmann, Bath vaccination of rainbow trout (*Oncorhynchus mykiss* Walbaum) against *Yersinia ruckeri*: effects of temperature on protection and gene expression, Vaccine 26 (2008) 1050–1062 https://doi.org/10.1016/j.vaccine.2007.12.029.
[16] G. Kurath, K.A. Garver, S. Corbeil, D.G. Elliott, E.D. Anderson, S.E. LaPatra, Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout, Vaccine 24 (2006) 345–354 https://doi.org/10.1016/j.vaccine.2005.07.068.
[17] J.M. Dijkstra, H. Okamoto, M. Ototake, T. Nakanishi, Luciferase expression 2 years after DNA injection in glass catfish (*Kryptopterus bicirrhus*), Fish Shellfish Immun 11 (2001) 199–202 https://doi.org/10.1006/fsim.2000.0303.
[18] T.C. Tonheim, J. Leirvik, M. Løvoll, A.I. Myhr, J. Bøgwald, R.A. Dalmo, Detection of supercoiled plasmid DNA and luciferase expression in Atlantic salmon (Salmo salar L.) 535 days after injection, Fish Shellfish Immun 23 (2007) 867–876 https://doi.org/10.1016/j.fsi.2007.03.015.
[19] N. Lorenzen, E. Lorenzen, K. Einer-Jensen, S.E. LaPatra, Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens, Dev. Comp. Immunol. 26 (2002) 173–179 https://doi.org/10.1016/S0145-305X(01)00059-3.
[20] M.K. Purcell, G. Kurath, K.A. Garver, R.P. Herwig, J.R. Winton, Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination, Fish Shellfish

Immun 17 (2004) 447–462 https://doi.org/10.1016/j.fsi.2004.04.017.
[21] N.O. Harun, T. Wang, C.J. Secombes, Gene expression profiling in naïve and vaccinated rainbow trout after *Yersinia ruckeri* infection: insights into the mechanisms of protection seen in vaccinated fish, Vaccine 29 (2011) 4388–4399 https://doi.org/10.1016/j.vaccine.2011.04.003.
[22] M. Wilson, E. Hsu, A. Marcuz, M. Courtet, L. Du Pasquier, C. Steinberg, What limits affinity maturation of antibodies in Xenopus–the rate of somatic mutation or the ability to select mutants? EMBO J. 11 (1992) 4337–4347.
[23] E. Bengtén, M. Wilson, Antibody repertoires in fish, Pathogen-host Interactions: Antigenic Variation V. Somatic Adaptations, Springer International Publishing, 2015, pp. 193–234 https://doi.org/10.1007/978-3-319-20819-0.
[24] V.M. Barreto, Q. Pan-Hammarstrom, Y. Zhao, L. Hammarstrom, Z. Misulovin, M.C. Nussenzweig, AID from bony fish catalyzes class switch recombination, J. Exp. Med. 202 (2005) 733–738 https://doi.org/10.1084/jem.20051378.
[25] L. Kato, A. Stanlie, N.A. Begum, M. Kobayashi, M. Aida, T. Honjo, An evolutionary view of the mechanism for immune and genome diversity, J. Immunol. 188 (2012) 3559–3566 https://doi.org/10.4049/jimmunol.1102397.
[26] B.G. Magor, Antibody affinity maturation in fishes—our current understanding, Biology 4 (2015) 512–524 https://doi.org/10.3390/biology4030512.
[27] N. Jiang, J.A. Weinstein, L. Penland, R.A. White, D.S. Fisher, S.R. Quake, Determinism and stochasticity during maturation of the zebrafish antibody repertoire, P. Natl. Acad. Sci. U. S. A. 108 (2011) 5348–5353 https://doi.org/10.1073/pnas.1014277108.
[28] S.L. Kaattari, H.L. Zhang, W. Khor, I.M. Kaattari, D.A. Shapiro, Affinity maturation in trout: clonal dominance of high affinity antibodies late in the immune response, Dev. Comp. Immunol. 26 (2002) 191–200 https://doi.org/10.1016/S0145-305X(01)00064-7.
[29] K.D. Cain, D.R. Jones, R.L. Raison, Antibody–antigen kinetics following immunization of rainbow trout (*Oncorhynchus mykiss*) with a T-cell dependent antigen, Dev. Comp. Immunol. 26 (2002) 181–190 https://doi.org/10.1016/S0145-305X(01)00063-5.
[30] J. Ye, E.S. Bromage, S.L. Kaattari, The strength of B cell interaction with antigen determines the degree of IgM polymerization, J. Immunol. 184 (2010) 844–850 https://doi.org/10.4049/jimmunol.0902364.
[31] J. Ye, I.M. Kaattari, S.L. Kaattari, The differential dynamics of antibody subpopulation expression during affinity maturation in a teleost, Fish Shellfish Immun 30 (2011) 372–377 https://doi.org/10.1016/j.fsi.2010.11.013.
[32] B. Star, A.J. Nederbragt, S. Jentoft, U. Grimholt, M. Malmstrom, T.F. Gregers, T.B. Rounge, J. Paulsen, M.H. Solbakken, A. Sharma, et al., The genome sequence of Atlantic cod reveals a unique immune system, Nature 477 (2011) 207–210 https://doi.org/10.1038/nature10342.
[33] D. Haase, O. Roth, M. Kalbe, G. Schmiedeskamp, J.P. Scharsack, P. Rosenstiel, T.B. Reusch, Absence of major histocompatibility complex class II mediated immunity in pipefish, *Syngnathus typhle*: evidence from deep transcriptome sequencing, Biol. Lett. 9 (2013) 20130044https://doi.org/10.1098/rsbl.2013.0044.
[34] E.S. Bromage, I.M. Kaattari, P. Zwollo, S.L. Kaattari, Plasmablast and plasma cell production and distribution in trout immune tissues, J. Immunol. 173 (2004) 7317–7323 https://doi.org/10.4049/jimmunol.173.12.7317.
[35] H.L. Saunders, A.L. Oko, A.N. Scott, C.W. Fan, B.G. Magor, The cellular context of AID expressing cells in fish lymphoid tissues, Dev. Comp. Immunol. 34 (2010) 669–676 https://doi.org/10.1016/j.dci.2010.01.013.
[36] M. Yamasaki, K. Araki, T. Nakanishi, C. Nakayasu, A. Yamamoto, Role of CD4+ and CD8α+ T cells in protective immunity against Edwardsiella tarda infection of ginbuna crucian carp, Carassius auratus langsdorfii, Fish Shellfish Immunol. 36 (2014) 299–304 https://doi.org/10.1016/j.fsi.2013.11.016.