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Diversification of Cytokines Across Vertebrate Immune System Evolution, Reproductive Efficacy and Tumor Escape

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Abstract

Cytokines have been identified and studied extensively in mammals, but little is known about their presence in other vertebrate groups. Although the divergence of cytokine subfamilies began before the fish-tetrapod split, much of the divergence within the subfamilies took place separately in different vertebrate groups. Many cytokines and their receptors, like most molecules of the immune system, tend to evolve rapidly, so that it has not been a simple task to isolate their ancestor genes. Cytokine homologs are found within jawless vertebrates, although no cytokine or cytokine receptor genes have been sequenced in cartilaginous fish except newly discovered IL-1β-like gene. Several cytokines, including IFNs, IL-1, IL-2, IL-6, IL-8 and TGF-β, have been identified in birds, reptiles, amphibians, and bonefish. During vertebrate evolution, this number grew dramatically, as well as the parallel growth number of regulatory cells and their function in the controlling of immune reaction. In lower vertebrates, cytokine network of the immune reaction consists mainly of the cytokines which are in mammals called the pro-inflammatory cytokines. Clearly defined, specialized and strong immunosuppressive cytokines have been only found in mammals, although the role of anti-inflammatory cytokines in lower vertebrates could be associated with the TGF superfamily. Even in birds, whose immune system is most similar to mammalian, cytokines like IL-4 and IL-10 have not been clearly identified. In conclusion, evolution of the adoptive immunity is associated with emergence of auto-immunity and alloimmunity/reproductive efficacy as by-products of self/non-self recognition. With auto-immunity and alloimmunity (especially in mammals) as new evolutionary forms of selection pressures, emergence of strong control mechanisms of immune reaction is not surprisingly. In this regard, diversification of cytokines through evolution and emergence of suppressive cytokines, as well as other control mechanisms of immune reaction, can be defined as another by-product of adoptive immunity. In addition, there are significant correlations between strenght of adoptive immunity and number of cytokines in different classes of vertebrates. Finally, these relations can be associated with anti-tumor immunity failure and source of different incidences of neoplasms in vertebrates.

Keywords: Cytokines, immunity, vertebrate, mammals, tumor, evolution

Evolutionary Diversification of Cytokines

Chemokines are small, inducible, structurally related proteins that guide cells expressing the right chemokine receptors to the sites of immune response. Albeit, there are described chemokines in bonefish and cartilaginous fish, as well as chemokine receptors in other jawed vertebrate, the phylogenetic analysis does not reveal any clear evidence of the orthology of lower vertebrates and human chemokines. The existence of chemokine receptors in the lamprey indicates that chemokines are apparently also present in the oldest vertebrate - *Agnatha* (1).

Albeit cytokines with vertebrate counterparts are likely to be present in invertebrates, no gene has been cloned that leaves a large gap in our knowledge of cytokine evolution. The functional analogues of vertebrate pro-inflammatory cytokines have been described in a variety of invertebrates. The analogy is based mainly on the crossreactivity of antibodies elicited against vertebrate cytokines, the sensitivity of invertebrate immunocytes to the action of vertebrate cytokines, and the responsiveness of vertebrate immune cells to invertebrate factors. However, without knowing the aminoacids or gene sequences of the putative invertebrate cytokine analogues, it has not been possible to demonstrate unequivocally a phylogenetic relationship between vertebrate cytokines and their invertebrate functional analogues. For example, a defence molecule from the earthworm *Eisenia foetida* (Annelidea) and the mammalian TNF-α perform similar functions, but they probably emerged independently during the evolution (2). In recent years, cytokines, which have been well characterised within mammals, have begun to be cloned and sequenced within non-mammalian vertebrates, with the number of cytokine sequences available from primitive vertebrates growing rapidly. The identification of cytokines, which are mammalian homologues, will give a better insight into where the immune system communicators arose and may reveal the molecules, which are unique to certain organisms.

LPS-treated fish monocytes produce a cytokine that is the homologue of IL-1 in mammals. This cytokine displays similar effects on immune and other cells as well. The structural similarity between fish IL-1-like molecule and mammalian IL-1 is approximately 50%. In order to be able to demonstrate these analogies, it is necessary to know that the similarity between mammalian IL-1α and IL-1β is about 25% (2,3,4). The LPS stimulated supernatant of both bird and amphibian monocytes, does not elicit IL-1-like activity, unlike mammalian lymphocytes. It is surpassingly due to the similarity of IL-1-like molecules in birds and mammals are approximately 60%.

These are convincing evidences corroborating the assumption that IL-1 and IL-1R probably emerged earlier than vertebrates and have been relatively conserved during the evolution (3,4):

1. IL-1 molecules of mammals and other vertebrate classes elicit interspecies crossreactivity with IL-1R;
2. Anti-IL-1 antibodies also display interspecies crossreactivity;
3. There is a relatively high similarity between mammalian and non-mammalian IL-1 like molecules;
4. Effects produced by IL-1 and IL-1-like molecules are identical or very similar on the cells of mammals, other vertebrates and even invertebrates.

Unlike IL-1, IL-2-like molecules stimulate T cell growth in species-specific manner, though some evidence reveals certain inter-species cross-reactivity of anti-IL-2R antibodies. For example, mammalian IL-2 administered before or after allotransplantation, did not affect allograft rejection or regeneration in amphibians (5). However, some studies have shown that intraperitoneal injection of human recombinant IL-2 can effectively modulate in vivo immune reactivity to thymus-dependent and thymus-independent type 2 immunogens in *Xenopus laevis*, but is less successful at affecting toad cells in vitro (6). Similar results were obtained by cultivating the lymphocytes of turtle with human IL-2. Eventually, IL-2 did not increase the fraction of turtle lymphocytes in mitosis even after days of repeated cultivation (7).

Although IL-2-like molecules have been identified in higher vertebrate classes only, a better understanding of IL-2 and IL-2R would still require a more detailed comparative investigation. Nevertheless, we are currently able to conclude that evolution of IL-2 has been characterized by higher polymorphism, i.e. lower degree of conservation as unlike IL-1 genes (8,9). Bird IL-2-like molecule and IL-2R are most similar to mammalian, and are known to strongly stimulate proliferation of CD4+ and CD8+ of bird peripheral lymphocytes (10). In addition, the mammalian IL-15 analogue has been discovered in birds, displaying similar effects as IL-2 (11).

No firm evidence has been found consistent with the fact that mammalian analogue IL-4 participates in the regulation of immune response in non-mammalian classes of vertebrates, unlike the analogues of IL-6 superfamily genes which have been isolated in all classes of these, except cartilagofish. Leukaemia Inhibitor Factor (LIF) and IL-6 genes in bonefishes show a low degree of evolutionary conservation, since the similarity between these and the mammals are less than 20% (12). IL-6-like genes have also been found in some species of amphibians *(Rana esculenta)*, but our knowledge remains scarce regarding the function of this cytokine family in amphibians (9). Bird

IL-6-like molecule is known to be synthesized and secreted after stimulation of the lymphocytes, and also to participate in the up-regulation of lymphocyte proliferation. Finally, the similarity between bird and mammalian IL-6 is approximately 35% (13).

Inoue *et al.* (14) found that some fish species have gene for IL-8. The same authors showed that the dogfish *(Triakis scyllia)* IL-8 sequence shared 50.5, 37.1 and 40.4-45.5% identity with the chicken, trout and mammalian IL-8 sequences, respectively.

Genes encoding anti-inflammatory cytokine IL-10 have not been identified in non-mammalian vertebrate classes, except for a small number of studies indicating presence of IL-10-like and IL-10R-like genes in birds, being clustered together with IFNR genes in the same way as in mammals (15).

To the surprise of many evolutionary immunologists, Yoshiura *et al.* (16) found that comparative genomic analysis showed a conserved synthesis within the IL-12 regions between *Fugu-fish* and human, indicating that the *Fugu* genes are orthologues for mammalian IL-12 encoding genes, respectively. The deduced amino acid sequences of the *Fugu* IL-12 subunits showed homology with mammalian IL-12 subunits (50.4-58.0% similarity). These studies have been the only evidence of IL-12 presence in non-mammalian vertebrates so far.

IL-15 is cytokine that has been identified only in birds and mammals. In birds, IL-15 is secreted by splenocytes in response to LPS and performs the function of pro-inflammatory cytokines, similarly to mammals (17).

TGF-β genes are probably evolutionary older than vertebrates, so the TGF-β gene superfamily is clearly ubiquitous among vertebrates. This cytokine is a strong inhibitor of the adoptive immunity across classes and species. A wide interspecies presence of TGF-β, high functional similarity and least 50% similarity between TGF-β genes of phylogenetically distant species, all indicate the relative conservation of these genes. The evolutionary conservation of TGF-β genes is not likely to be the result of their involvement in the regulation of immune response, but probably the consequence of other, also very important biological mechanisms, such as cell differentiation, maturation of oocytes (18), mechanisms controlling the formation of the embryonic axis in amphibians and probably in all classes of vertebrates (19). Finally, Paulesu *et al.*, (20) studies have shown that the production of cytokines like TGF-β by the feto-placental unit is not limited to mammalian species, since TGF-β can be secreted by the placenta of such viviparous squamate reptiles as *Chalcides chalcides*. The finding of this parallelism between reptilian and mammalian reproduction suggests that immunological mechanisms, possibly mediated by the secretion of cytokines, played an important role in the evolution of viviparity.

Molecular cloning and expression analysis of TNF-α in all classes of vertebrates reveal its constitutive expression and ubiquitous nature. The protein sequence deduced from *(Sparus aurata L.)* TNF-α gene shows a high degree of homology with the *Japanese flounder* TNF-α (65.6% identity and 78.9% similarity) and, more important, it is more homologous to mammalian TNF-α (41.1-48.6% similarity) than to TNF-β (36.0-43.5% similarity) (21). Although TNF superfamily plays important role in immune reaction in all vertebrates, the evolutionary conservation of the family, like in the case of TGF, is probably associated with the non-immune and co-immune mechanisms, like apoptosis, regeneration, tissue remodelling, metamorphosis etc.

The IFN family consisting of IFN-α, IFN-β, IFN-Ω, IFN-δ, IFN-κ, and IFN-τ is a large group of cytokines involved in the innate immune response against various microbes. While the IFN have not been found in invertebrates, the genes for IFN type I are expressed by almost vertebrates. Genes for IFN have been cloned from a variety of mammalian and avian species; however, IFN genes from cartilagofish have not been forthcoming. Despite the considerable advances in our understanding of teleost immunity, relatively few cytokine genes, including those for IFN, have been identified at the molecular level. In contrast, numerous studies have shown that following virus infection or exposure to double-stranded RNA, fish immune cells produce a soluble factor that is functionally similar to mammalian IFN. Also, there are reports considering the cloning and characterization of the IFN gene from the zebrafish (22). IFN type I comprise IFN-α secreted by leucocytes and IFN-β secreted by virus-infected cells, whereas interferon type II (IFN-γ) is secreted by the activated T cells and APCs. IFN-α and IFN-β are mainly involved in the inhibition of virus replication, while the role of IFN-γ in the immune response is the up-regulation of class I, class II and TAP/LMP genes transcription. IFN type II is much more difficult to identify in lower vertebrates. Although there are no consistent reports about the presence of this cytokine in fishes and reptiles, it has been found that the lymphocytes of many fish species react to human IFN-γ, with increased MHC expression, which is indicative of the presence of IFN-γ-like the factor which performs a similar function like in mammals. IFN-γ-like factor has been isolated from birds, and found to elicit similar interspecies crossreactivity with mammalian lymphocytes, although the similarity between bird and mammalian IFN type II is as low as 35% (23).

Cytokine Network in Pregnancy and Tumor Sufferers

The implantation of the human embryo is a multiple paradox, because the feto-placental unit shows the characteristics of successful transplant, parasite and tumor. Many different mechanisms are included in the priming

of endometrial environment for successful implantation, but one of most important events is the establishing of optimal cytokine network. Much evidence has suggested that decidual cytokines and chemokines play a very important role in the embryo implantation, endometrial development, and trophoblast growth and differentiation by modulating the immune and endocrine systems. Sex steroid hormones, cytokines and chemokines mediate the close correlation between the trophoblast and the decidua (24,25,26).

Endometrial macrophages, NK cells, dendritic cells, lymphocytes, neutrophils and other decidual cells build a specific cytokine network that regulates the activity of decidual immune cells. The domination of Th2 cells and Th2 type of cytokines (IL-4, IL-6, and IL-10) are preferentially found in healthy, normal decidua. *In vitro* studies have shown that this microenvironmental conditions inhibit the proliferation of cytotoxic and Th1 cells. Finally, the secretion of anti-inflammatory cytokines and the inhibition of T and NK cell activation and proliferation, strongly suggest that decidual immune cells create an immunosuppressive environment on the decidual-trophoblast junction (24,25,26).

A defective or excessive trophoblastic invasion, as well as an aberrant cytokine network of the decidua, probably can result in the complications of pregnancy, as early spontaneous miscarriage, preeclampsia and the growth retardation of vascular origin. In this case, decidual cytokine network is associated with pro-inflammatory cytokines, which involve Th1 cell activation. Pro-inflammatory cytokines such as IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, TNF-α and IFN-γ promote the inflammation by increasing the permeability of blood vessels and consequently increases the access of immune cells that can reject the placenta as an allotransplant (27,28,29,30).

A large number of experiments and studies have clearly shown that cytokines secreted from the tumor cells or immune cells infiltrating and surrounding the tumor tissue affect the final outcome of the anti-tumor immune reaction by exerting immunomodulatory/suppressive effects. While it is true that cytokine network of tumor microenvironment contains the pro-inflammatory and immunostimulatory cytokines, it seems that their effects are largely subject to the effects exerted by modulatory and suppressive cytokines, such as IL-4, IL-6, IL-10 and TGF-β (31,32). The serum concentration of IL-6 is quite often enhanced in patients suffering from malignant diseases. In patients with malignant lung carcinoma, the serum concentration of this cytokine is several times higher than in patients with an obstructive disease or acute infection (33). In melanoma suffering patients who show a good response to treatment, the concentration of this cytokine is only two times higher in relation to the healthy control. Also, in terminal patients with disseminated melanoma the serum concentration of IL-6 is even 11 times higher in relation to the healthy control (33).

Higher values of IL-10 in sera have also been frequently reported in various tumor sufferers. At the same time, a significantly lower concentration of IL-2 in the tumor tissue and sera of these patients has been reported. The intralesional treatment with IFN-γ was associated with tumor regression and down-regulation of IL-10 mRNA. The importance of IL-10 secretion by tumor cells is evidenced by the fact that five-year survival of the patients suffering from IL-10 non-secreting tumors is approximately 90%, while as little as 15% of five-year survivors was reported in patients with IL-10 secreting tumors. As well as secreting IL-10, malignant tumors also may induce IL-10 and immunosuppressive prostaglandin E2 (PGE2) production by monocytes. The reduced activity of $CD8^+$ and $CD4^+$ cells in TIL population can be enhanced by IFN-γ and TNF-α, which strongly inhibit the synthesis and the secretion of anti-inflammatory cytokines, such as IL-10 (32,33,34).

IL-10 is one of most important cytokines that prevents the class I molecules expression at the cell surface. Terrazzano *et al.* (35) investigated the IL-10 effects in a human lymphoblastoid cell defective for TAP1 and TAP2 genes after TAP1 and TAP2 genes transfection. In this experimental system, the down-regulation of antigen presenting/processing machinery was observed in TAP transfected cells in the presence of IL-10, whereas the processing and presenting of peptides in IL-10 non-treated transfected cells was almost normal.

IL-10 and TGF-ß, is a cytokine that exerts multiple effects on antigen presentation, B and T cell proliferation, cytokine production, and monocyte/macrophage function. IL-10 prevents up-regulation of CD80/86 expression during macrophage activation and down-modulates the expression of a broad range of cytokines in peripheral blood mononuclear cells (PBMC), including IFN-γ, IL-2 and TNF-α (33).

Tumor cells incubated or transfected with IL-10 had decreased but peptide-inducible expression of class I, decreased sensitivity to class I restricted CTL, and increased NK sensitivity. IL-10 signal inhibit the TAP dependent translocation of peptides to the ER, resulting in the accumulation of immature class I molecules in the endoplasmic reticulum and subsequently a low expression of cell surface class I molecules. This finding is explained by a down-regulation of expression of TAP1 and TAP2, observed in IL-10 transfected tumor cells. IL-10 is the first example to demonstrate that a cytokine can decrease the expression and function of the TAP1 and TAP2 molecular complex and, in more general terms, the first example of a cytokine with an inhibitory effect on class I-mediated peptide presentation (36,37).

A high level of TGF-β was also found in the sera of patients suffering from progressing tumors, with a much lower level of this cytokine secreted by regressing tumors. Disseminated processes as well as processes resistant to

conventional therapy are most frequently associated with a high TGF-β concentration in the sera. Consistent with these data, the addition of anti-TGF-β antibodies to autologous lymphocyte/tumor cells co-cultures increased the frequency of cultures showing effective anti-tumor response immune (31).

Conclusion

The oldest classes of vertebrates are characterized by a small number of cytokines, probably tracing the origin from the regulatory factors whose primary role was probably not associated with immune mechanisms. During vertebrate evolution, this number grew dramatically, as well as the parallel growth of the function in the controlling of immune reaction. The largest number of cytokines (especially anti-inflammatory) we can see in mammals, as well as expanded cytokine network controlling strong killer machinery of adoptive immunity. This evolutionary ability can be repercussion of selection pressures of auto-immunity and alloimmunity/reproductive efficacy and might be source of anti-tumor immunity failure (24,25).

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