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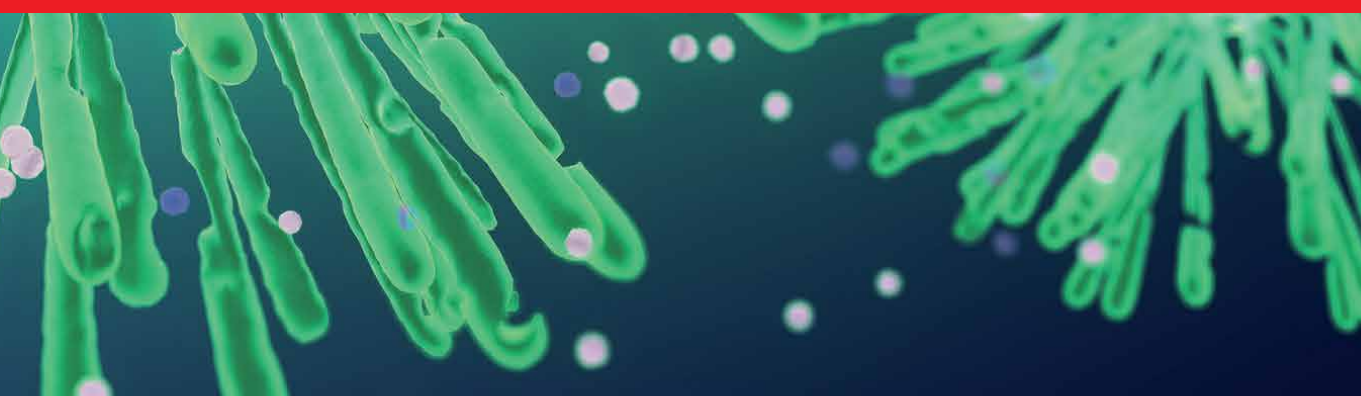
Antimicrobial Immune Response

Edited by Maria del Mar Ortega-Villaizan and Veronica Chico



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Published in London, United Kingdom







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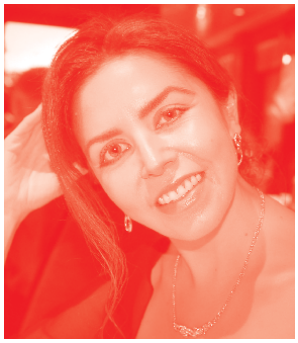

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http://dx.doi.org/10.5772/intechopen.87657
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First published in London, United Kingdom, 2021 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, 5 Princes Gate Court, London, SW7 2QJ, United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Antimicrobial Immune Response
Edited by Maria del Mar Ortega-Villaizan and Veronica Chico
p. cm.
Print ISBN 978-1-83968-782-2
Online ISBN 978-1-83968-783-9
eBook (PDF) ISBN 978-1-83968-784-6

Meet the editors

Dr. Ortega-Villaizan obtained a degree in Biological Sciences from the University of Alicante, Spain, in 1995, and a Ph.D. in Agricultural Sciences (Fish Population Genetics) at the University of Tohoku, Japan, in 2005. In her postdoctoral period, she began working as a researcher at the Institute of Research, Development, and Innovation in Healthcare Biotechnology in Elche (IDiBE), University Miguel Hernandez (UMH), Spain. Dr. Ortega-Villaizan was working as a postdoctoral researcher in what would be her current research line, which is the search of therapies and prophylactics against viral pathologies in aquaculture, specifically in the context of the fish immune response. In 2014, she was granted an ERC Starting Grant, which commenced in 2015 at the IDiBE-UMH. Dr. Ortega-Villaizan was leading her own laboratory to investigate the crosstalk between red and white blood cells in the context of viral infections and DNA vaccine immunizations to elucidate the role of red blood cells in the fish immune response. Since 2018, she has been an assistant professor in the Department of Biochemistry and Molecular Biology, UMH. Her current research interests are the red blood cells of fish and their involvement in fish immune response and as target cells in the search for therapies or prophylactics against fish viral infections.

Dr. Chico obtained a degree in Biology at the University of Alicante, Spain, in 2003, and a degree in Biochemistry at Miguel Hernandez University (UMH), Spain, in 2005. She acquired a Ph.D. in Molecular and Cell Biology with a focus on Fish Immunology and Viral Prophylaxis at the Institute of Research, Development, and Innovation in Healthcare Biotechnology in Elche (IDiBE), University Miguel Hernandez (UMH), Spain in July 2010. In 2012, she began working as a postdoctoral researcher at the Department of Organism Biology and Comparative Physiology, Uppsala University, Sweden. Her work was focused on innate immunity and hematopoiesis in crustaceans. Since 2015, Dr. Chico has been a postdoctoral researcher at the IDiBE-UMH, Spain. She works with Dr. Maria del Mar Ortega-Villaizán's research group at the IDiBE´s research subgroup of Antiviral and Antimicrobial Strategies. Her current research interest is the evaluation of the innate and adaptive immune response to viral pathogens in aquaculture in the context of the role of red blood cells as cell mediators in the fish immune response.

Contents

Preface

The environment comprises a variety of infectious microbial agents. Many of them can cause pathological disorders and even death in organisms exposed to them if they multiply uncontrollably. However, organisms can control infections caused by pathogens thanks to the existence of the immune system. The immune system is a set of biological processes that prevents an organism from infectious diseases [1]. In vertebrates, the immune system is divided into the innate and the adaptive immune systems. The innate immune system is the most ancient form of defense. It is the first mechanism to respond to infections and the main defense mechanism in invertebrates [2]. It is characterized as non-pathogen-specific and does not provide specific long-lasting immunity to the host [3]. The components of the innate system comprise the physical barrier (the skin), molecular effectors (complement system, antimicrobial peptides, and cytokines), and immune cells (granulocytes, monocytes, macrophages, and natural killer cells) [3].

The innate immune system has certain specificity in the recognition of pathogens through pattern recognition receptors (PRRs). These receptors are expressed in many cell types, and they are strategically located throughout cells. PRRs are in cell membranes where they mediate recognition of extracellular pathogens, and in endosomes and cytoplasm where they detect intracellular pathogens. PRRs recognize small molecular motifs characteristic of pathogens called pathogen-associated molecular patterns (PAMPs) [4], which are conserved through evolution. There is a variety of PAMPs, for example, bacterial flagellin, bacterial lipopolysaccharides (LPS), peptidoglycans, or nucleic acid variants from viruses [double-stranded RNA (dsRNA) or nonmethylated viral 5'-C-phosphate-G-3′ (CpG)-containing DNA] [4]. The activation of PRRs with their PAMPs activates the signaling networks that modulate the expression of cytokines such as type I interferon and antiviral proteins to protect the organism against infections [5].

In addition, vertebrates possess the adaptive immune system, which consists of a specific immune response based on immune memory against recurrent pathogens [6]. B and T lymphocytes are principally responsible for the specificity of the adaptive immune responses [7]. This system is highly specific and can discriminate between self- and non-self-cells. Both the innate and adaptive immune systems do not act separately; they are completely integrated to protect the organism against the attack of pathogens [1].

Nowadays, the immune system of higher vertebrates like mammals is being more studied in depth in comparison with the immune system of lower vertebrates such as teleosts. The immune system of teleosts is physiologically comparable to that of higher vertebrates, despite certain differences such as the fact that the main haematopoietic organ of teleosts is the head kidney, as they do not have bone marrow (the main haematopoietic organ in mammals) [8]. Apart from that, teleosts possess a less complex adaptive immune system compared to higher vertebrates and therefore rely heavily on innate immune responses to face continuous pathogen attacks. Teleosts reside in extremely distinct environments from those in which mammalians have evolved, so it is not misbegotten that aquatic vertebrates have

	Teleost	Mammals
Immunoglobulins	IgM, IgD, and IgT/Z	IgM, IgG, IgA, IgD, and IgE
Class switch recombination	No	Yes
Antibody affinity maturation	Deficient	Very high
Memory T cells	Low	Very high
Lymphoid tissues	Spleen, thymus, and head kidney	Spleen, thymus, and bone marrow
Macrophages, monocytes, NK-like cells, and granulocytes	Yes	Yes
RBCs actively participate in immune response	Yes	No

Table 1.
Comparison of key immune response elements between teleosts and mammals, modified from [8].

many immunological differences from terrestrial vertebrates [9]. As to the innate immune response, in lower vertebrates it is similar to that of higher vertebrates, and the main cell types involved in this response are macrophages, monocytes, NK-like cells, and granulocytes [10]. A difference between the immune system of teleosts and mammals is the red blood cells (RBCs), since in contrast to mammalian RBCs, the RBCs of fish are nucleated. Nucleated RBCs, characteristic of fish, amphibians, reptiles, and birds, have been recently stated as multifunctional cells because in addition to being involved in gas exchange and transport, they also can actively participate in the immune response to several pathogens [11]. Regarding the adaptive immune response, it is known that teleost B lymphocytes do not possess the same repertoire of immunoglobulins as humans [12], and they also have different antibody affinity maturation and lymphocyte proliferation processes [13]. **Table 1** compiles some of these and other differences.

This book presents current investigations regarding the humoral and/or cellular mechanisms responsible for the induction of antiviral and antibacterial immune responses in different immune-reactive organs, for example, skin, lungs, gut, bone marrow, kidney, spleen, blood, liver, and reproductive organs.

Additionally, this book provides the reader with an overview of the mechanisms that have been the target of interest in terms of therapeutics or prophylactics against viral or bacterial infections. The purpose of this book is to show an up-to-date revision of the antimicrobial mechanisms triggered across different animal species, from lower to higher vertebrates.

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Acknowledgments

The editors would like to thank the staff at IntechOpen, particularly Commissioning Editor Jelena Germuth and Author Service Manager Maja Bozicevic, for their contributions to the editorial process.

Chapter 1

Immune System of Fish: An Evolutionary Perspective

Sujata Sahoo, Husne Banu, Abhinav Prakash and Gayatri Tripathi

Abstract

Fishes are the most successful and diverse group of vertebrate animals, first appeared during Devonian period. Despite of certain differences, the immune system of fish is physiologically similar to that of higher vertebrates. The heterogenous group of fishes are the apparent link between innate immunity and the first appearance of the adaptive immune response. Importantly, fishes have immune organs homologous to that of mammalian immune system. In comparison to higher vertebrates, fishes live free in their environment from the early embryonic stage and during that time mostly they are dependent on non-specific immune system for their survival. In the fishes, non-specific immunity is the fundamental defense mechanism, therewith acquired immunity also plays key role in maintaining homeostasis by activation though a system of receptors proteins, which identify pathogen associated molecular pattern typical of pathogenic microorganism includes lipopolysaccharides, peptidoglycans, DNA, RNA and other molecules that are typically not present on the surface of multicellular organism. There are several external factors like environmental factors, biological factors, stress and internal factors like genetic makeup, age and sex, maternal effect etc. can affect immunological defense capabilities of the fishes.

Keywords: Fish immune system, innate immunity, adaptive immune response, defense mechanism, environmental factor

1. Introduction

Evolution has brought many genetical and physiological innovations in animal phyla including alteration in immune mechanism. Immune system of fish is a subject which provides unique insight towards evolution of defense system in vertebrate lineage. Fish as an earliest vertebrate in evolutionary history, has a distinct pattern of immune morphogenesis in comparison to other higher vertebrates. They are heterogeneous group of poikilothermic animals which include jawless fish (e.g., Lamprey) and jawed fish of class Chondrichthyes and Osteichthyes. Their physiology and immune system development vary among them and it is highly influenced by environmental parameters, unlike warm blooded vertebrates. External parameters like photoperiodism, temperature and oxygen concentration of water influence development and functioning of both innate (e.g., Complement, lysozyme activity) and adaptive immunity (e.g., IgM concentration) in fish [1]. Apart from

environmental influence some of the variations are inherited and evolved via genetic alterations. It appears mostly in the adaptive immune mechanism especially in form of genetic recombination process which is the key of diversification of repertoires of lymphocyte based antigen recognition receptors [2]. The role of various genes and organs involved in defense mechanism of jawed and jawless fishes are discussed here in order to provide complete information on progress or innovation in fish immune system.

2. Immunity of agnathans

Despite the diversification, many features of fish immunity i.e., immune gene expression, inflammation, wound healing, antigen pattern recognition receptors, signaling and trafficking of lymphocytes remains conserved across the vertebrate linage. These functions are mostly played by the cellular and humoral factors of the immunity. The agnathans lack hematopoietic organs i.e., spleen, thymus or kidney but they have unique strip of medullary tissue present throughout the length of trunk called Immune body [3]. The dedicated organs for immunity have not been so far detected but some of the area of lamprey typhlosole and renal folds carry hematopoietic stem cells and lymphoid like cells and differentiated cells including thrombocyte, granulocyte, monocyte, and lymphocyte like cells have also been detected [4]. The humeral factors like antimicrobial peptide coding genes i.e., cathelicidin genes has been detected in Atlantic hagfish (*Myxine glutinosa*) [5]. Other innate immunity related genes such as reactive oxygen species modulator I and Peroxiredoxin coding gene and NFκB inhibitor gene are being detected in immune body and other tissues which indicate for the presence of a well-developed innate defense mechanism [6]. The lamprey oral gland also found to secrete many defenses related functional proteins i.e., interferon-induced lethality protein-19 and disintegrins. The components involved in complement activation pathway have been detected in Lamprey [7]. The homologous components like C3, mannose-binding lectin (MBL), and MBL-associated serine proteases (MASP) of the lectin pathway and factor B of the alternative pathway have been identified from lamprey and/or hagfish but the cytolysis process in unique in terms of serum protein named "lamprey pore-forming protein" (LPFP).

The signature molecules of adaptive immunity i.e., MHC genes, T cell receptors and B cell receptors are absent in primitive agnathans but in place there are lot of leucin rich repeats coding sequences indicating an alternative pathway of adaptive immunity [8]. Some of the research has found specific agglutinin-based memory for antigen recognition in Atlantic lamprey and agglutinin secreting cells in the intestine. The lamprey has unique lymphocytes expressing orthologous genes encoding B-cell signaling components i.e., PU.1/Spi-B. The classical VDJ gene recombination process which is required for creating diversifies repertoire of Ig based B cell receptors in higher vertebrates are absent in Agnathans. The Lymphoid like cells has found to express complex LRR carrying molecule called variable lymphocyte receptors (VLR) which under goes subsequent assembly through an entirely novel genomic mechanism in which large banks of LRR cassettes are used to build the 'diversity' region of the receptor molecules [8]. The basic composition of these VLR includes a conserved signal peptide, an N-terminal LRR (LRRNT), followed by nine variable and highly diverse LRRs, a connecting peptide, a C-terminal LRR (LRRCT), and a conserved C terminus (GPI)-anchor site and a hydrophobic tail. Upon antigen induction there is a marked proliferation of hematopoietic lymphoid cells and increased VLR protein receptors for variable antigen detection.

In adult lamprey the VLR gene expression has been detected in typhlosole, opistonephros, supra-neural body and blood. In contrast the pharyngeal regions of larvae or embryos are found to express VLR genes especially in oral tentacles and the gill filaments [9].

3. Immunity of osteichthyes

As per the cellular organization and physiologic requirement there are variations in pattern of immune system ontogeny in different group of fishes. There are many similarities between fish and human immune system but unlike human they have a resilient innate immunity which helps them to survive and adopt to the adverse condition inside water. Fishes do not have bone marrow and lymph nodes but head kidney plays a major role in hematopoiesis as well as direct antimicrobial activity through melanomacrophage centers (MMC). Apart from anterior and middle kidney, thymus and spleen are two important lymphoid organs present in fish [10]. The development pattern of fish lymphoid organs is variable according to the type of fish but we will discuss some of the well-known discoveries related to ontogeny of fish immune system.

The kidney (head and middle), thymus and spleen are the largest lymphoid organ in teleost fishes. The development sequence of lymphoid organ varies between freshwater and marine water fish species [11, 12]. In case of freshwater teleost e. g. carp, tilapia and trout, kidney is the first lymphoid organ to develop and spleen is the last organ. Lymphoid organs of marine fish develop differently in order of kidney, spleen and thymus respectively. In marine water teleost fishes, such as cobia (*Rachycentron canadum)*, Flounder (*Paralichthyus olivaceaus*), Sea bream (*Sparus aurata*), yellow tail (*Seriola deumerili*) and red sea bream (*Pagrus major*) the anterior kidney is the first lymphoid organ to appear followed by spleen and thymus [13, 14]. But in both cases thymus is the first organ to have lymphoid cells followed by kidney and spleen.

3.1 Kidney

In teleost fish, kidney functions similar to bone marrow in the vertebrates and is the largest site of hematopoiesis [11]. Immune cells are present over entire kidney whereas anterior or head kidney has the highest concentration of developing B-lymphoid cells [15]. The anterior kidney is aglomerular and has hematopoietic function [16] and unlike higher vertebrates, it is principal organ for phagocytosis, antigen processing, formation of IgM and immune memory through melanomacrophage centres [17]. In fish, the head kidney serves as an important endocrine organ, homologs to adrenal gland in mammals and release corticosteroids and other hormones [18]. Furthermore, anterior kidney is the major site for antibody production.

Anterior/head kidney is the initial common site for hematopoietic stem cells (HSC) development and differentiation. At early hatching condition rudimentary pronephric kidney use to carry undifferentiated precursor cells even in the absence of any blood islands which are believed to be the first site of pluripotent stem cell formation in mammalian yolk sac. Comparison with human immune system reveals that after migration of precursor cells from fetal liver and spleen, pro-myeloid cell formation occurs in bone marrow for life time and this is why anterior kidney of fish is similar in action to bone marrow of higher vertebrates [19].

In zebrafish a well-developed kidney can be found at 72 hours post fertilization (hpf) but hematopoietic cells appear at 96hpf [20] However this timeframe for appearance of hematopoietic cells may be different in different fishes (**Table 1**).

Species	Appearance of lymphoid organ	Appearance of hematopoietic cell	Appearance of lymphocytes	References
Zebra fish	72hpf	>96hpf	3wpf	[20]
Rainbow trout	<8dbh	5dbh	5dph	[21]
Seabream	<1dph	5dph	54dph	[22]
Channel catfish	NK	<3dph	<7dph	[23]
Common carp	1dph	NK	6dph	[24]

Hpf-hours post fertilization, wpf-week post fertilization, dbh- days before hatch, dph-days post hatch, NK-not known.

Table 1.
Histogenesis of fish kidney.

By gradual differentiation immature precursor cells form cords, an aggregated form of more differentiated HSCs surrounded by blood vessels. These sinusoidal blood vessels are lined by fibroblastic reticular cells. Further development from pronephric to mesonephric kidney supports for the formation of erythroblast, myeloblast and lymphoblast.

3.2 Thymus

The lymphoid cells which are actually major immune blood cells initially are not differentiated in the head kidney. Thymus is the most important lymphoid organ which is found in all vertebrates including chondrichthyes and the osteichthyes but an exception in case of Lamprey and Hagfish which are known to be the primitive vertebrates. However, research for the presence of thymic analogue in lamprey has revealed Thy-1 reactivity which is mainly associated with thymus and Tcell development, has been captured in different tissues including typhlosole, opisthonephros, liver, external gill openings in larval lamprey [25]. Unlike mammals where thymus appears to carry and develop precursor cells migrated from bone marrow for T cells formation, in fish thymus is the first organ to be lymphoid. In fact, undifferentiated cells are found to be migrating from kidney to thymus through collagen fibers of pharyngeal septum during early developing stage of Turbot [13].

Thymus is present near gill arch and is closely associated with the pharyngeal epithelium internally facing towards head kidney. In zebrafish thymus appear as primordial outgrowth of pharyngeal epithelium at 54 hours post fertilization (hpf) (**Table 2**) and a developed thymus carry electro-lucent epithelial cells and mature lymphocytes [20]. The morphology of thymus varies in age dependent manner from species to species and within species. In carps, thymus alters from triangular to irregular shape and even the cortex as well as medulla changes their position. The distinct cortico-medullary junction is not present in all fish. The recombination activating genes (*rag*), which are responsible for rearrangement of immunoglobulin gene and T-cell receptor genes in immature B and T lymphocyte respectively are often used for histological localization of premature thymus. In zebra fish, the *rag1* gene expression at 92hpf distinguishes *rag1*+ cortex and *rag1*- medulla of thymus. Before this period *ikaros* gene which is responsible for lymphocyte differentiation is expressed in thymus at 72hpf [26].

Thymus of teleost is a bilobed homogenous organ placed in a dorsal projection in the epithelium of the operculum cavity and it is lined by mucus tissue of pharyngeal epithelium in structure that surrounds the lymphoid bark tissue is the characteristic

Species	Appearance of lymphoid organ	Appearance of hematopoietic cell	Appearance of lymphocytes	References
Zebrafish	54hpf	65hpf	3dpf	[20]
Rainbow trout	5dbh	1dh	3dph	[21]
Seabream	22-29dph	29-47dph	47dph	[22]
Channel catfish	NK	NK	5dph	[23]
Common carp	3dpf	NK	4-5dpf	[24]

Hpf-hours post fertilization, wpf-week post fertilization, dbh- days before hatch, dpf- days post fertilization, dph-days post hatch, NK-not known.

Table 2.
Histogenesis of fish Thymus:

of the fish thymus [27]. Thymus in the fishes has frequent record of variation in morphology due to the absence of cortico-medullary junction [28]. So, in many species it is not possible to differentiate between cortex and medulla that is found in higher vertebrates [29]. The involution of thymus in fish is more dependent on hormonal cycles and seasonal variations than on the age [18]. Teleost's thymus is much similar to mammalian in which erythrocytes, neutrophils and granulocytes are found in spleen whereas lymphocytes are major cell type found in thymus [18]. Thymus produces T lymphocytes involved in stimulation of phagocytosis, allograft rejection and antibody production by B cells [29].

3.3 Spleen

In teleost, spleen functions as major secondary immune organ, plays major role in the clearance of blood borne antigens and immune complexes in splenic ellipsoids and in the antigen presentation and initiation of adaptive immune response [30]. The size of spleen in fish is widely used as simple measurable immune parameter with potential role in immune response against parasite infections [31].

Spleen is the third important hematopoietic organ which originates in form of mesenchymal cell aggregate surrounded by blood capillaries. It is the third organ to be lymphoid but for a long time it carries erythroid cells only. The expression of Hox11 transcript factor which helps in survival of precursor splenic cells indicates splenic primordium appears during 5 dpf at left anterior gut portion of zebra fish [32], whereas it in rainbow trout it is found at 3dph (**Table 3**). The ellipsoids which are involved in plasma filtration and blood borne antigen trapping, appears at 3 months after hatching of zebrafish. These ellipsoids have narrow lumen which runs through reticular cells and macrophages.

3.4 Appearance of Ig + cells

There is no clear-cut development pattern of Ig + cell in fish but mature B cells are found earlier in freshwater fish in comparison to marine fish. In Atlantic halibut (*Hippoglossus hippoglossus* L.) appearance of first Ig positive cell take time up to 66 dph in kidney (**Table 4**) [33]. Head kidney seems to be the major organ for B cell maturation and IgM production except in zebra fish where pancreas first gets Ig + detection [34]. At 10 dpf Ig transcripts can be located in pancreas of zebra fish and later on (19 dpf) in kidney. In rainbow trout cytoplasmic Ig (cIg) can be detected on 12 dbh followed by surface Ig on 8 dbh [36]. In contrast surface

Species	Appearance of lymphoid organ	Appearance of hematopoietic cell	Appearance of lymphocytes	References
Zebrafish	4dpf	30dpf	3 month	[20, 32]
Rainbow trout	3dph	NK	6dph	[21]
Seabream	12dph	NK	54dph	[22]
Channel catfish	NK	NK	5dph	[23]
Common carp	5dpf	NK	8dpf	[24]

hpf-hours post fertilization, wpf-week post fertilization, dph-days post hatch, days post fertilization, NK-not known.

Table 3.
Histogenesis of fish spleen:

Species	Appearance of Ig + cells	Organ	References
zebrafish	7 dpf	Whole fish	[34]
	10 dpf	pancreas	
Common carp	2 wpf	head kidney	[35]
Rainbow trout	cIg 12 d pre-hatching	head kidney	[36]
	sIg 8 d pre-hatching	head kidney	
Atlantic halibut	66 dph	kidney	[33]

hpf-hours post fertilization, wpf-week post fertilization, dpf- days post fertilization, dph-days post hatch.

Table 4.
Ontogenesis of Ig + cells.

Ig (sIg) is detected earlier (2 wpf) than cytoplasmic Ig + cells (4 wpf) in carp kidney. All investigations indicate that appearance of Ig + cells and immunocompetence development may show variation in time due to temperature and other external factor influence [35].

3.5 Other tissues

Apart from the major hematopoietic organ, there are additional lymphoid tissues in different organs of fish. Expression of *Ikaros,* which is a gene specific for lymphoid cell differentiation, is marked to be present in bilateral patches of brain at 24–96 hpf, heart, intestine and testes [37]. Fish do not have typical lymphocyte accumulation site which is so called Peyer's patches (PP) in mammals but few macrophage-like cells and leukocytes are found in gut. However, mucosa-associated lymphoid tissue (MALT) of fish can be found in different forms like gut associated lymphoid tissue (GALT), Gill associated lymphoid tissue (GIALT), Skin associated lymphoid tissue (SALT), nasal-associated lymphoid tissue (NALT), and the recently discovered buccal and pharyngeal MALTs. GALT is known to carry immunoglobulin expressing cells such as T and B cells in intraepithelial lymphocyte and lamina propria respectively. A maximum number of intraepithelial leukocytes are found in proximal and distal gut portion but their distribution and concentration vary according to species, diet, temperature and other external influence [38]. In teleost hind gut carries most of the Ig positive lymphocytes and the macrophages

associated with gut looks different comparison to kidney and spleen macrophage. These differential immune cells are found at 14 dph in *0reochromis.mossambicus* (Tilapia) and get fully matured during 7 weeks which is quite earlier in comparison to GALT maturation in *Burbus conchonius* (during 20 weeks). Such gut lymphoid cells can be seen during 8dpf in zebrafish whereas in rainbow trout are found in gut epithelial region during 13 dph. Occasionally at the age of 54dpf few lymphocytes like cells are found in gut and skin of sea bream which is a marine fish [35]. Unlike the mammals' fish like Rainbow trout secretes IgM, IgT/IgZ [37] and channel catfish secrets IgD in mucus [38]. These MALT associated Igs specific transcript expression can be detected at 4dpf in whole carp embryo but developed IgM and IgZ are found later during 4–6 weeks post-fertilization.

4. Fish innate immunity

Non-specific immunity found in all living organisms and is the first line of defense against all pathogens, also plays an important role in the activation of adaptive immune response. The cells of the innate system recognize and respond to pathogens in a generic way. It also possesses memory as the host evolves its innate immune components based on evolutionary experience of its ancestors encountering similar pathogens [39]. Innate immunity is commonly divided into three compartments: surface barrier, humoral factors and cellular factors. As the first line of defense, it is not surprising that the majority of the broad-spectrum parameters of innate immunity are highly conserved across species and taxa. In all jawed vertebrates, the innate immune system features a rapid defensive response towards invading pathogens and tissue damage. However, it cannot provide well-directed, specific protection from individual pathogens or long-term immunological memory.

4.1 Surface barrier

Mucus, skin, gills and gastrointestinal (GI) tract acts as first line of barrier to any infection. Layer of mucus present in skin, gills and GI tract entraps microorganisms by continuously sloughing and inhibits colonization. Mucus of fish is toxic to certain microorganism due to presence of some humoral factors. The rate of mucus production increases in response to infection or by physical or chemical irritants [40].

The epidermis of fish skin is composed of non-keratinized living cells and the integrity of these cells plays vital role in maintaining osmotic balance and excluding microorganisms. Rapid healing is also observed in epidermis of fishes [41].

Large surface area of delicate gill epithelium considered as important route of pathogen entry. The gills are protected by mucus production and highly responsive epithelium resulting in hyperplasia, frequently seen in various gill infections. Phagocytic cells line the branchial capillaries, lymphoid cells on the caudal edge of the intrabranchial septum.

GI tract is lined by mucus membrane and also the digestive enzymes, bile and low pH of stomach provides an extremely hostile environment for pathogens.

4.2 Humoral factors

There is array of soluble substances which have protective function which inhibits the growth of microorganisms and neutralizes the enzymes on which pathogen depends. The classification of humoral parameters is commonly based on their pattern recognition specificities or effector functions.

4.2.1 Growth inhibitors

Growth inhibitors acts either by depriving microorganism of essential nutrients or by interfering with their metabolism. Transferrin occurs in serum, exerts a bacteriostatic and fungistatic effect. Transferrin is a protein with high Iron (Fe) binding capacity, which is an essential element for growth of microorganism and deprives them of iron [42]. Pathogenic bacteria may produce their own chelating agents like siderophores to overcome this defense mechanism and hyperferremic activity acting as a counter response has been demonstrated in some fish species. Transferrin is also an acute phase protein invoked during an inflammatory response to remove iron from damaged tissue [42] and an activator of fish macrophages [43]. Interferons are another virus inducible cytokine which induces the expression of Mx and other antiviral proteins [44]. Grinde (1989) studied the antibacterial effect of two lysozyme variants (Types I and II), purified from the head kidney of rainbow trout, on seven Gram-negative bacterial fish pathogens [45]. INFα and β are cytokines with a nonspecific antiviral function that is based on the inhibition of nucleic acid replication within infected cells. Interferons are potent activator of downstream antiviral defenses and the type I Interferons (IFN-α and β) induces expression of wide range of Interferon stimulated genes (ISG) inducing Mx, Viperin, ISG 15, PKR leading to enhanced antiviral state. Type II interferons (IFN- γ) promotes Th 1 cell responses produced primarily by CD4 + Th 1 cells and NK cells. Th 1 cell provide defense against intracellular pathogens such as viruses and bacteria by inducing apoptosis restricting cell proliferation during viral infection. Fish IFN also modulates cytokines and chemokines expression and is potent inducer of proinflammatory cytokines such as IL-1, IL-6, IL-12 and tumor necrosis factor (TNF).

4.2.2 Enzyme inhibitors

Pathogens produce enzymes in order to penetrate and obtain nutrients from their hosts. Tissue fluids and serum of vertebrates contains many enzyme inhibitors which are thought to defend body against autodigestion and also plays an important role in neutralizing enzymes produced by pathogens. Fish plasma contains a number of protease inhibitors, principally α1-antiproteinase and α2-macroglobulin (α2M). Many bacteria produce proteolytic toxins which digest host tissue proteins as a source of amino acids. An important protease produced by *A. salmonicida* is resistant to rainbow trout α1-antiproteinase but is inhibited by α2M [46]. The difference in α2M activity between two different trout species (rainbow trout and brook trout) has been found to correlate with their resistance to *A. salmonicida* infection [46] suggesting that α2M may play a role in defense against furunculosis.

4.2.3 Lysins

Various lytic enzymes either in single or in combination may cause lysis of pathogenic cells. Lysins in fishes include complement, lysozyme and antimicrobial peptides. Lysozyme is the most studied innate response in fish which act on the peptidoglycan layer of bacterial cell walls resulting in the lysis of bacteria [47]. Lysozymes synthesized both in liver and extra hepatic sites and are present in mucus, lymphoid tissue, plasma as well as in other fluids and is also expressed in a wide variety of tissues [48] and involved in a comprehensive defense mechanism, such as bacteriolysis, opsonization, as well as restricted antiviral and antineoplastic activity, as found in higher vertebrates [49].

Studies of the integument and integument secretions of fish [50] have demonstrated an important role of antimicrobial peptides in host defense against viruses and bacteria [51]. These peptides are found in mucus, gills and liver tissue of teleost fishes [52] and include liver expressed antimicrobial peptides (LEAP), Defensins, Piscidins, and Cathelicidin.

Complement system is the biochemical cascade that helps or complements the ability of antibiotics to clear pathogens from the host. Complement system plays major role in the link between both innate and adaptive immune responses that allows an integrated host defense to pathogenic challenges [53]. Complement system plays multiple functions like mediating inflammatory vasodilation, lysis of bacterial cells and infected cells, opsonization to foreign particles to enhance phagocytosis, clearance of apoptotic cells and also in alternation of molecular structure of viruses. The bactericidal activity of complement has been reported in many fishes [54]. Complement system gets activated by three pathways- the classical pathway, which is triggered by antibody binding to the cell surface [55], the alternative pathway, which is independent of antibodies and is activated directly by foreign microorganisms, and the lectin pathway, which is activated by the binding of a protein complex consisting of mannose/mannan-binding lectin in bacterial cells [56].

4.2.4 Agglutinins and precipitins

Mucosal or serum agglutinins and precipitins are lectins like C-type lectins and pentraxins. The C-type lectins have binding capacity for different carbohydrates like mannose, N-acetyl glucosamine or fucose in the presence of Ca ions, and the interaction between carbohydrate binding protein and carbohydrate leads to opsonization, phagocytosis and activation of the complement system [57]. Mannose binding lections (MBL) are the most studied lections which show specificity for mannose, N-acetyl glucosamine, fructose and glucose. Lections, with various carbohydrate specificities, have been isolated from the serum of several fish species [58]. Pentraxins (C-reactive protein, CRP and serum amyloid protein, SAP) are lectins, which are present in the body fluids of both invertebrates and vertebrates and are commonly associated with the acute phase response [59]. Pentraxins are pattern recognition proteins that are important component of acute phase response to infection or injury. Some best known pentraxins are C-reactive protein (CRP) which is known to bind with phosphoryl choline present on many microbial cell wall and Serum amyloid protein (SAP) binds to phosphoethanolamine, glycans and also known to bind LPS of Gram-negative bacteria [60].

4.3 Cellular factors

The cellular components of the fish's innate immune system consist of many different types of cells such as monocytes/macrophages, granulocytes as mast cells/eosinophilic granule cells, and neutrophils, dendritic cells, and natural killer cells (NK cells). When an innate immune cell encounters and recognizes a pathogen through its pathogen-associated molecular pattern (PAMP), the immune cells get activated and can participate in several responses depending on their cell subtype, including phagocytosis and subsequent destruction of pathogens [61].

4.3.1 Macrophages/monocytes

Macrophages are the first cells to arrive and respond to the site of infection. Macrophages are derived from hematopoietic progenitor cells (immature cells), which differentiate through circulating monocytes or via tissue-resident

macrophages namely kuffer cells in liver, glial cells in brain, etc. [62]. Macrophage differentiation is controlled by engagement of the colony-stimulating factor 1 receptor (CSF1R) [63] first identified in the elephant shark (*Callorhinchus milii*) genome [64]. Macrophages in teleost play a role in both the innate and adaptive immune systems and are vital players during inflammation and pathogen infection. In the innate immune system, macrophages destroy pathogens through phagocytosis, reactive oxygen species (ROS) and nitric oxide (NO) production, and the release of several inflammatory cytokines and chemokines, similar to mammalian macrophages [65]. Similar to mammals, teleost fish also have functionally distinct macrophages [66]. In teleost fish species, M1 (classically activated macrophages) are characterized by the production of pro-inflammatory cytokines such as TNFa and IL-1b and production of ROS and NO [67], and these cells may rapidly kill pathogens by engulfment and production of toxic reactive intermediates, phagolysosomal acidification, and restriction of nutrient availability [66]. Whereas M2 are alternatively activated macrophages and are mainly associated with immunosuppression, trauma, and anti-inflammatory cytokines such as interleukin (IL)-10 [68].

4.3.2 Phagocytic B cells

Phagocytosis mediates the primary action of the teleost immune system, is the central effector mechanism of innate immunity, and also plays an essential role in linking the innate and adaptive immune responses in vertebrates. Phagocytosis is an endocytic process of phagocytes by which other cells or particles, including microbial pathogens, are ingested or engulfed to form phagosomes and phagolysosomes, followed by the destruction of the invader or the continued processing of antigenic information, eventually initiating adaptive immunity in vertebrates [69]. Classical phagocytosis is mainly versed by "professional" phagocytes, like macrophages/monocytes, neutrophils, and dendritic cells. Moreover, some "amateur" phagocytes such as epithelial cells and fibroblasts can also internalize antigens particulate to a much lower degree compared to professional phagocytic cells [70]. It is very well known that B cells in all vertebrates are functional antibody-secreting cells (ASCs) for producing specific antibodies in response to certain invading foreign antigens and those them play vital roles in adaptive immunity [71]. It was a long-held paradigm that B cells are non-phagocytic cells, even though evidence has been reported that CD5+ B-cell lymphoma could differentiate to macrophage-like cells [72]. In 2006, for the first time, it was reported that B cells derived from teleost fish and frog are competent of phagocytic and bactericidal activity through the formation of phagolysosome, which was previously only identified in professional phagocytes [73]. Moreover, teleost fish, this novel phagocytic capability of B cells has also been notified into other vertebrates like reptiles [74], mice, and humans [75]. IgM+ B cell is the most abundant immunoglobulin present in the serum of teleost fish and was first reported in rainbow trout (*Oncorhynchus mykiss*) and catfish (*Ictalurus punctatus*) for their characteristic phagocytic and bacteria-killing abilities [73]. In the subsequent study, in rainbow trout the IgM−/IgT+ B-cell subset, which uniquely secretes IgT, gets identified, capable of phagocytic and microbicidal activity [76]. In recent years, the phagocytic B cells of teleost fish have been identified from about ten teleost fishes but were only focused on IgM+ B-cell subsets due to the deficiency of specific mAbs against IgT or IgD in these fish species [69]. The phagocytic activity of IgM+ and IgT+ B cells could be significantly increased after incubation with antiserum or complement-opsonized target particles [77]. The regulatory mechanisms of interleukin IL-6 and IL- 10

are recognized in the phagocytic activity of teleost IgM+ B cells [78], where IL-10 could enhance the phagocytosis of IgM+ B cells in flounder [79]. A number of B Cell receptor (BCR) like mIgM, CD79a, CD79b [80], and other cell receptors, such as Toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs), which are common pattern recognition receptors (PRRs) of professional phagocytic cells, may also be involved in B-cell phagocytosis [81]. The concurrence of complement and phagocytic B cells indicates the essential importance of B cells in the linkage of innate and adaptive immunity. The highly variable phagocytic abilities for the IgM+ B cells to ingest different microbial particles were also reported in zebrafish (*Danio rerio*), lumpfish (*Cyclopterus lumpus* L.), half-smooth tongue sole (*Cynoglossus semilaevis*), large yellow croaker (*Larimichthys crocea*), and Japanese flounder (*Paralichthys olivaceus*) [82]. Teleost phagocytic B cells study is still at an early stage, and more efforts are required for further detailed investigation of immune functions in teleosts.

4.3.3 NK cells

Non-specific cytotoxic (NCC) cells are akin to mammalian natural killer (NK) cells, but they do not contain cytoplasmic granules like NK cells and having pleomorphic clefted nucleus with little cytoplasm with different killing mechanism [83]. They share several similarities, mainly the competent lytic cycle, the target cells for lysis, recognition of target cell, and the effecters to lyse the infectious microorganisms [84]. In almost all fish species, NK cells or NK-like functional activities have been described [85]. Cells with NCC activity are primarily present in the blood, lymphoid tissues, and the gut. NCC needs to physically contact target cells without membrane fusions or fragmentation [86]. The smallest leucocyte NCC targets various cells, including tumor cells, transformed cells, virus-transformed cells, and protozoa parasites [87]. The killing is spontaneous, non-specific, and does not require any apparent induction period. NCCs are reported to be most active in the head kidney of teleosts, but spleen and peripheral blood leukocytes (PBL) also demonstrate cytolytic abilities [88]. The NCC activities are influenced by age, strain, temperature, stress, and activity are more pronounced when specific responses are less active.

4.3.4 Stromal cells

Stromal cells are connective tissue cells of organs that act in a supportive capacity to the parenchymal cells performing specific organ functions. During the last decade, when the complexity and function of stromal cells were revealed in immune functions, the stromal cells were considered "non-hematopoietic immune cells" before that it was merely known for providing a structural framework upon which hematopoietic immune cells could function [89]. The growing evidence suggests that non-hematopoietic stromal cells exhibit a capacity for diverse cell intrinsic and extrinsic immune function in many non-lymphoid tissues, including the intestine, where it plays multiple immune responses inflammation at this mucosal site [90]. Intestinal stromal cells are non-professional immune cells that recognize bacteria and other cells via TLR or NLR and modulate T-cell function [91]. Stromal cells have various mechanisms to directly sense bacterial contact, respond rapidly on contact with pathogen proving protective immune response, and respond to cytokine signals from the epithelium and thus amplify both protective and potential deleterious immune responses [92].

4.3.5 Red blood cells

Unlike mammalian cells, fish red blood cells are nucleated and contain organelles in their cytoplasm [93]. The nucleated fish red blood cells are well known for gaseous exchange but recently their new biological role in immune response has been reported [94]. Nucleated red blood cells (RBCs) of fish contain the transcriptional and translational machinery necessary to produce characteristic molecules of the immune system to respond against various infectious agents and play an active role in maintaining homeostasis of the fish immune system [95]. The nucleated RBC are reportedly involved in both innate and adaptive immune responses in fish [96]. Nucleated RBCs are able to phagocytose, acts as antigen-presenting cells [97, 98], recognizes pathogen associated molecular pattern (PAMPs) by specific pathogen recognition receptors (PRRs), modulate leukocyte activity, release cytokine-like factors [99, 100] and also induces interferon in fish [101]. The expression of immune-relevant genes in RBC had shown a wide repertoire of TLRs in *Salmo salar* and *Oncorhynchus mykiss*, which allow them to respond to both bacterial and viral infections [95]. However, to know more about the involvement of RBC in immune response, more studies are required and several researchers are working on it.

4.3.6 Intestinal cells

The gastrointestinal tract cells function in digestion and maintain immune homeostasis to protect the body from potentially harmful microbes and induce a tolerogenic response to innocuous food, commensals, and self-antigens. Fish have local mucosal defense in the gut to sample antigens and produce local immunoglobulin responses [102]. Leucocytes are abundantly present in the fish gut's lamina propria and intestinal epithelium [103]. The indication of specific antibody secretion in the fish intestine comes after intestinal or immersion immunization of various fish species, which were rarely detectable after systemic immunization [104]. Immunoglobulins (Ig) produced in the intestine are a result of local synthesis was get confirmed after intravenous administration of radiolabeled Ig, which never reached the mucosal secretions. Ig isotype (IgT) is specialized for mucosal immunity, and in trout fish, the IgT response to a gut parasite is restricted to the intestine [102]. The Polymeric immunoglobulin receptor (pIgR), an essential component of mammalian mucosal immunity, has also been described in few fish species [105]. Ig + B cells and Ig-T cells are abundantly present in fish's gut, but limited data is available regarding their functional relevance [106].

The fish intestine, especially the posterior segment, is immunologically active and armored with various immune cell types, including B cells, macrophages, granulocytes, and T cells.

4.3.7 Fish gill

Diseases associated with gill damage, cause substantial losses in the aquaculture industry not only through an increased mortality rate among fish but also through impaired growth and also by increased treatment and sanitation cost. Damage to gill tissues is specially characterized by inflammation and increased epithelial cells hyperplasia or hypertrophy. A gill epithelium of salmonids has higher number of MHC class II positive cells [107] whereas low number of macrophages like cells has been detected in gill epithelium of presumably healthy salmonid fish [108].

5. Conclusions

Fish immunity although similar to other higher organisms, there is differences owing to their natural habitat. Fish are a heterogeneous group of poikilothermic animals consist of jawless fish and jawed fish of class Chondrichthyes and Osteichthyes. Their physiology and immune system development vary among them and is highly influenced by environmental parameters, unlike warm blooded vertebrates. Here we highlighted the development of immune system in different class of fish along with components of immune system.

Acknowledgements

We acknowledge director, ICAR-CIFE, for providing necessary funding and facilities.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

Host-Microbial Relationship: Immune Response to Microbial Infections with or without Medication

Faustina Pappoe and Samuel Victor Nuvor

Abstract

Immune responses of the host to any infectious agents vary in controlling the pathogens. The process begins by the entry of microorganisms into the host to initiate host immune response to understand the type of microorganisms and react accordingly for possible elimination of the organisms. In some cases the host co-exists with the pathogens or unable to effectively deal with them leading to disease condition. Thus, the pathogens establish, multiply and cause disease. The review considered the mode of acquisition of infection, pathogenesis and immune responses to microbial infection. Other areas included the enhancement of immune responses to control infection, immune responses of the host under drug treatment and the control of microbial infection. The understanding of the relationship between infectious microbes and the host immune system leading to protective immunity or disease state will give much information about treatment and controlling of microbial infection in our environment.

Keywords: immune response, host-microbial, pathogens, infectious agents, drug treatment

1. Introduction

Several human diseases are caused by pathogenic microorganisms which are diverse and are divided into four major groups namely bacteria, viruses, parasites and fungi [1]. Thus, different pathogens cause varied diseases. Members in each group were classified into subgroups based on unique characteristics they possess [2]. Bacteria were differentiated based on their staining properties due to variation in the cell wall components and those without cell wall, hence there are gram-positive, gram-negative, acid-fast and cell wall defective bacteria. These were subdivided by their shape (spherical and rod-shaped bacteria), growth requirement (e.g. aerobic and anaerobic) among others [3]. Viruses have DNA and RNA with each kind having either single-stranded or double-stranded nucleic acid. These were further classified by the presence or absence of an outer envelope, shape, size and other characteristics [3, 4]. The parasites included protozoa, helminths and arthropods. Unlike helminths and arthropods, which were multicellular, the protozoans were unicellular and conveniently classified by their mode of locomotion.

The protozoans included amoebas, ciliates, flagellates and apicomplexans. The helminths were classified according to their shape: nematodes (roundworms) and platyhelminths (flatworms and tapeworms). The arthropods were also considered as vectors of pathogens mainly viruses and bacteria [3, 4]. Finally, the fungi were made up of unicellular forms (*Saccharomyces cerevisiae*) and multicellular forms (molds). The molds were subdivided into hyphae and conidia forms [3].

Generally, pathogenic microorganisms are either primary/true pathogens or opportunistic pathogens. The primary pathogens were those capable of causing diseases in the host irrespective of the host's immune system. Thus, they cause diseases in immunocompetent and immunocompromised individuals and persons with slight imbalances of the immune system. However, the opportunistic pathogens mostly included the normal flora and only cause diseases in immunocompromised individuals as well as when they occur in parts of the body that were not natural to them [5]. When infection occurs, there is interaction between the host immune system and the pathogens. The outcome involved either immune control towards the infection or disease development with pathological manifestations due to the inability of the host immune responses to effectively deal with the pathogens [5, 6]. Understanding the immune responses to microbial infections with or without medication is necessary in the management, control and prevention of infectious diseases. This chapter focuses on the mode of acquiring infections, pathogenesis and immune responses to microbial infection, enhancement of immune responses to control infection, immune responses of the host under drug treatment and preventing microbial infection.

2. Modes of transmission of infectious diseases

Infection is the multiplication of pathogens in or on the body of the infected host whereas disease is the impairment in the normal function of the host because of damage to the host's cells by the infection [7, 8]. Thus, for infection or disease to occur, the pathogens must attach to or enter the body of the host, multiply, evade the immune responses, cause damage to the host cells and spread to new hosts. In some individuals, the disease is symptomatic while in others, it is asymptomatic. The time interval between infection and appearance of the first clinical sign or symptoms of disease was known as incubation period and this was the time the infection can be spread without the person knowledge [7]. The incubation period is influenced by several factors such as dose of a pathogen, route of inoculation, rate of replication of infectious agent, host susceptibility and immune responses. Hence, incubation period varies among diseases. For instance, non-typhoidal *Salmonella typhi* has incubation period of 10 to 14 days, that of *Bordetella pertussis* is 7 to 10 days, among others [4, 9]. The incubation period is followed by prodromal period whereby microbial agents continuously multiply and the host begins to experience general signs and symptoms of illness which are mostly general to be associated with a particular disease. The signs and symptoms were due to activation of the immune system [5]. After the occurrence of the prodromal period is the period of illness during which individual feels extremely sick and can easily spread the infections followed by the period of decline. The declining period is associated with the controlling of the replication of the pathogens resulting in lessening of the signs and symptoms of the disease. Thus, individuals feel better at this state. This period is followed by the period of convalescence where microbial replication stops, and the person fully recovers from the disease. However, in some cases, individuals who have recovered fully can still spread the infection in the environment [5]. What it means is that the immune responses are strong against the pathogens

to prevent development of clinical manifestations but are unable to destroy the pathogens in the body so the person harbors and spread the infection in the environment. Those individuals are called carriers. A typical example is a person with typhoid fever. The pathogen was continuously shed in the feces to the external environment hence the infection could be acquired through ingestion of fecally contaminated food or drinks [10, 11]. Human immunodeficiency virus (HIV) and hepatitis B and C carriers could spread the infection through blood products and body fluids [12, 13]. Another example was a tuberculosis infected person with mild clinical presentations, but persistent cough could spread the infection through air before the disease was diagnosed [14]. Vertical transmission through transplacental infection was also possible (e.g. *toxoplasma*, *rubella*, *cytomegalovirus*, *Herpes* simplex, and other organisms including *Treponema pallidum*, HIV, *parvovirus*) [15]. There were other infectious diseases such as anthrax, balantidiasis, toxoplasmosis, taeniasis and rabies that were zoonotic and could be acquired from animals [16, 17]. Insect vectors such as female Anopheles, ticks and sandflies could also help spread the infections including malaria, babesiosis, rickettsiosis and leishmaniasis respectively [18–21]. In summary, infectious diseases can be acquired in several ways including horizontal means such as touching contaminated surfaces, direct skin contact, body fluids, airborne, vector borne, and ingesting raw/undercooked meat. Other mode of transmission includes fecally contaminated food and water and vertical transmission among adult and children.

3. Pathogenicity of microbial infection

The ability of a microbe to cause disease is known as pathogenicity and the degree or extend of the pathogenicity is termed virulence. Virulence varied from mild to severe with varying virulent factors that directly or indirectly play a role in pathogenicity and virulence [22]. Hence, some pathogenic microbes are avirulent causing diseases only occasionally, moderately virulent that cause mild diseases while others are highly virulent causing diseases with severe clinical presentations. For a microbe to cause a disease, the pathogens must attach to and/or enter the host body with the help of virulent factors and colonize [23–25]. The main attachment and entry sites for microorganisms include the skin, conjunctiva, alimentary, respiratory and urinogenital tracts. Some microbes attached to and sometimes penetrate the host body surfaces such as the skin and cells (nucleated and non-nucleated) using adhesins (proteins) located on the surface of the pathogen [26]. The adhesins bind to specific host receptors, which could be transmembrane glycoproteins or extracellular matrix proteins. Others entered directly through open surfaces like skin wounds, through inhalation, a vector such as bites from infected arthropods, mammals like dogs involved in rabies cases and piercing by contaminated devices such as needles [25, 27–30]. The conjunctiva is mostly infected by the fingers, face towels, flies that settle there among others. *Chlamydia trachomatis* and *Neisseria gonorrhea* were sexually transmitted pathogens that commonly cause conjunctivitis in neonates [31] who acquire the infection from infected cervix during normal birth. Not much about the pathogenesis of *C. trachomatis* is known. However, *C. trachomatis* is an intracellular pathogen and inhibits phagosome and lysosome fusion when it is phagocytosed thereby evading host immune defenses [32]. Mucosal surfaces of the respiratory tracts have immune mechanisms and cells that prevent pathogen attachment and colonization. Hence, some invading pathogens such as *Streptococcus pneumoniae* could attach to epithelial cells only when the mucocillary and other immune mechanisms were defective [33]. However, some pathogens have strong attachment structures. For instance, *Bordetella pertussis* has fimbriae and produces

a kind of protein called filamentous haemagglutinin A (FHA) which enable the pathogen to attach to the epithelial cells of the bronchia and the lungs [34] thereby disrupting the ciliary activity leading to their multiplication, colonization and host tissue damage. *Mycobacteria tuberculosis* is phagocytosed by alveolar macrophages in which most die. However, some survive and continue to replicate until the macrophages die leading to their release, where some reinfect other cells and some enter the blood and lymph circulations; carried to other parts of the body [35]. The pathogens of the gastrointestinal tract cannot be overlooked. *Helicobacter pylori* is an important intestinal pathogen that was associated with chronic gastritis, peptic ulcer and gastric cancers [36]. It possesses flagella and adhesins for attachment to the gastric mucosa. It produces several vital enzymes most notably urease which enable the pathogen to survive in the gastric environment for colonization. Urease acts on urea and degrades it to form ammonia and carbon dioxide. Ammonia neutralizes the acid in the stomach making the environment favorable for its survival. Moreover, *H. pylori* produces toxins such as vacuolating cytotoxin, and cytotoxin-associated gene encoded by the vacA and cagA genes respectively [37]. These toxins/proteins induce intense inflammatory responses leading to damage to the host tissues. The immune response is unable to eliminate this pathogen hence the use of antibiotics for their eradication. Another example is *Enterohemorrhagic Escherichia coli* (EHEC) serotype O157:H7, which is a true human pathogen and causes bloody diarrhea, hemorrhagic colitis (HC) and life-threatening complication such as the hemolytic-uremic syndrome (HUS). This pathogen is resistant to destruction by the gastric acid and so passes the acidic barrier and get to the recto-anal junction (RAJ) where it attaches tightly and forms attaching and effacing (A/E) lesions on the RAJ mucosal epithelium for colonization [38]. It produces Shiga-like toxins which when enters the circulation leads to HUS. Additionally, *Giardia lamblia,* noninvasive parasite possess sucking disc for attaching tightly to the epithelium surface of the small intestine leading to inflammatory responses as well as malabsorption due to destruction of the villi. The attachment is also aided by lectins, which are found on its surfaces and the flagella aid in motility [4].

Regarding the urinogenital tract, it is mostly sterile as a result of frequent flushing by urine, hence most invaded pathogens are flushed out and do not get access into the system. However, certain pathogens like *Neisseria gonorrhea* when invaded were able to colonize the tract [39]. This results in the infection of mainly the cervix, urethra, and rectum. The mouth, nasopharynx and the eye may also be affected. The virulent factors included pili, which enable it to attach firmly to the epithelial cells of urogenital sites, OPA proteins (adhesives) and IgA proteases [4]. It worth noting that women frequently get urinary tract infection than men because of the difference in the anatomical structure. Thus, men have longer urethra than females.

4. Microbial infections and the corresponding immune response towards their elimination

Infection of the host by the pathogens responses in the host with initial reaction of the innate immune response followed by the adaptive immune responses. Infection involving bacteria is associated with various mechanisms to evade or survive the host immune response. Some of the bacteria form capsules, complex structures which present many diverse antigenic targets to the host body surface [40, 41]. The capsules are effective at hiding many bacterial surfaces and preventing opsonization to enable them circulate systemically within the body. Some of these bacteria involved in capsule formation included *Streptococcus pneumonia,*

Haemophilus influenzae, Escherichia coli, and *Neisseria meningitides* which rely extensively on its capsule to prevent antibody and complement deposition on its surface [42] thereby avoiding opsonization and phagocytic clearance.

Viruses also evolve a number of techniques for evading the immune responses by avoiding complement system through rearrangement of epitopes in their surface proteins. The *measles* virus prevent antibodies binding to haemagglutinin to initiate complement by the classical pathway [43] presumably because the antigenic epitopes were so spaced that effective bridging cannot be obtained between them. Human Immunodeficiency Viruses were able to bind to cells through complement receptors after fixing complement and also Dengue virus which could enter cells through Fc receptors after having bound antibody [44]. Other organisms such as *Herpes virus saimiri, Trypanosoma cruzi* and *Schistosoma mansoni* [45], captured complement control proteins to change their function [46]. However, the immune response to microbial pathogens relies on both innate and adaptive components and they work together to eliminate the pathogens. Macrophages and dendritic cells were found in all body tissues, serving as sentinels in wait for pathogens and respond to variety of chemotactic agents that were shed as a result of infection [47]. The cells bind the pathogens via phagocytic receptors that initiated the cytoskeletal rearrangements and membrane trafficking for phagocytosis [48, 49]. Other innate cells like neutrophils, basophils, eosinophils and NK cells contributed together in clearing of the pathogens through phagocytosis, cytotoxicity, and the release of cytokines to enhance their activities in eliminating the pathogens [50]. The adaptive immune cells are made up of B and T lymphocytes, including γδT cells, T reg cells and Th17 cells. Microbial antigens are taken up by antigen-presenting cells in the peripheral tissues and delivered to the lymph nodes or spleen through the lymph or blood, respectively. They are therefore recognized by these adaptive cells and differentiate specifically into several types of effector cells, depending on the class of pathogens they recognized. The differentiation of lymphocytes into a particular effector-cell type and their localization to the site of infection were regulated by the innate immune system, generally in the form of cytokines and chemokines [51]. The effector cells therefore exhibit their function through cytotoxity as well as the release of cytokines which together aid in destroying the pathogens.

5. Enhancement of immune response to control infection

Antigenic features of microbes known as pathogen-associated molecular patterns (PAMPs) are recognized by Pattern Recognition Receptors (PRRs). These involve Toll-like receptors (TLRs), NOD-like receptors (NLRs), AIM2-like receptors (ALRs) and RIG-I-like receptors (RLRs) and stimulation with ligands promptly potentiated the production of proinflammatory cytokines and chemokines [52] which facilitated the clearing of bacterial infections. There was significant reduction in the number of *Haemophilus influenzae* and *Moraxella catarrhalis* bacteria recovered from the nasopharynx through intranasal inoculation of monophosphoryl lipid A in mice [53]. The use of PRR ligands for *Staphylococcus aureus* adjuvants vaccine formulated with a TLR7 agonist and adsorbed onto alum adjuvant (4CT7-Staph) conferred about 80–90% protection against four different *Staphylococcal strains* [54]. NOD-like receptors were also important for clearing a variety of bacterial infections, including *Salmonella Typhimurium, S. flexneri, Pseudomonas aeruginosa, and B. pseudomallei* [55]. Most often, *B. pseudomallei* induces NLRC4-dependent pyroptosis which restricts intracellular bacterial growth. However, the activation of NLRP3, upregulates IL-1β, promoted the replication *of B. pseudomallei* and recruited excessive neutrophils to the lung leading to tissue damage [56].

Identifying small molecules that selectively activate NLRP3 inflammasome and prevent cytokine secretion may also be promising new therapeutic strategy.

Most bacterial killing are enhanced by autophagy activity in response to cellular stresses, including hypoxia, energy loss, and nutrient deprivation. This process provided a mechanism for the adaptation to starvation and regulated cellular metabolism and homeostasis [57], therefore play a major role in homeostatic maintenance. The use of autophagy as innate immune mechanism for the clearance of intracellular pathogens [58] enhances the efficient immune responses in dealing with pathogens. Alternatively, bacterial clearance could also occur through LC3-associated phagocytosis (LAP), which was mediated through single-membrane phagocytic vesicles that contain engulfed pathogenic bacteria including *Escherichia coli, S. Typhimurium, Mycobacterium marinum,* and *B. pseudomallei* [59]. These were transiently coated with LC3-II and sirolimus, an mTOR inhibitor, that increased the colocalization of the bacteria with LC3 in phagosomes, thereby augmenting phagosomal maturation and further phagocytosis [60]. Also treatment of macrophages with AMG548, a p38 inhibitor, promoted the clearance of *M. tuberculosis* by inducing autophagy [61]. The host response to hypoxic conditions created by bacterial infections regulated by hypoxia-inducible factor (HIF) which [62] drove the expression of proinflammatory cytokines that mediated macrophage aggregation, invasion, and motility thereby enhancing the intracellular killing of the bacteria during replication [63, 64].

Again, macrophages and neutrophils produced reactive oxygen species (ROS) and reactive nitrogen species (RNS) molecules that acted as a defense mechanism to trigger the clearance of the phagocytosed microorganisms [65]. However, an imbalance in the production and elimination of ROS is associated with human diseases.

6. Drug treatment regime in microbial infection and the interaction with immune response

The treatment of any infections targets the clearing of the pathogens involved and allows the immune system to develop and fully functions. Therapeutic strategies for the treatment of microbial infections have mainly relied on the antibiotics that target pathogenic proteins, DNA, RNA, or cell wall synthesis. In some cases, not all the pathogens are cleared and some may resist clearance. In Tuberculosis (TB) infection, effective drugs have been available for decades, but the disease remained a major infectious disease at global level [66, 67]. This might be due to the emergence of *Mycobacterium tuberculosis* (Mtb) strains showing resistance to some of the most commonly used effective drugs: isoniazid and rifampicin [67]. These multi-drug resistant Mtb strains (MDR-TB) were responsible for 0.49 million cases of tuberculosis, mostly in India, China and the Russian Federation [67]. The interaction between Mtb infection in an immunocompetent host led to latent TB infection, with no signs or symptoms of active disease [68]. This involves the critical role of host innate and adaptive immune responses in the control of Mtb infection. The intrinsic ability of host responses to contain Mtb replication while preventing the development of the typical tissue damage, formed the hallmark of active TB [69]. There was therefore the persistence and a certain degree of replication of Mtb in host tissues in a dynamic equilibrium with the host, which in most cases lasted for lifetime [70, 71]. However, the immune responses that involve phenotype of immune cells with their chemokines and cytokines secretions responsible for the consequences at local level remains to be determined. Eventually, the critical role of the host immune response in the control of Mtb replication, or emergence of active disease instead depend on many factors and

may be assisted by drug therapy or microbial modulation of the immune system. For humans, these interactions could be infection with pathogenic microbes or vaccination [72]. Vaccination with Bacillus Calmette-Gue'rin (BCG), an attenuated strain of *Mycobacterium bovis,* protected against tuberculosis (TB), but its effects on the immune system extended far beyond specific protection against TB [73]. BCG vaccination has been shown to afford nonspecific protection against infection by a number of pathogens, including *Schistosoma mansoni* and *Listeria monocytogenes* [73]. The appearance of carbapenem-resistant *Enterobacteriaceae* had also affected the therapeutic benefit of the carbapenem class of antibiotics, which were reserved as a last-line defense [52, 74, 75].

Drug-resistant viral strains has also compromised the effectiveness of treatment, or even lead to its failure. Drug-resistant viruses occurred as a result of mutation at high frequencies of the viral RNA or DNA [76]. Their genotypes could be advantageous in hosts where the drug was present and could become the dominant genotypes in such hosts [77]. Influenza virus also developed resistance to oseltamivir drugs through mutations and there might be possible exchange of genetic information between resistant and susceptible viral strains [78]. The therapeutic options against HIV-1 include more than 20 drugs through their action mechanisms. These targeted to four different points of the viral replication cycle such as the entry of the virus into the cell, inverse transcription, the integration of viral genetic material into the cell nucleus, and maturation of virions [79]. This phenomenon has been associated with the high replicative capacity of the virus and the high error rate in the transcription of its genetic material. These might be due to the presence of specific mutations resulting from pharmacological pressure and suboptimal viral suppression under a treatment scheme [80]. Herpes virus infection depended upon viral inhibition of several cell functions including the turning off of host protein synthesis, inhibition of mRNA splicing, blocking presentation of antigenic peptides on the cell surface and apoptosis [81]. Treatment of HSV-infections with nucleoside analogs was very common but the development of drug-resistant virus from immunosuppress patients with prolonged exposure to the antiviral agent has been established [82–84]. Mutations of the herpes viral Thymidine kinase (TK) and DNA polymerase (DNApol) occurred and involved in mechanisms of resistance to acyclovir and penciclovir [85, 86]. The development of point mutations by the pathogens to survive drugs as well as the host immune response involve various factors associated with the infection. In some cases, less aggressive chemotherapeutic regimens substantially reduce the probability of onward transmission of resistance without significant changes in host pathology [87, 88]. In contrast, high dose aggressive treatment in controlling the resistant populations were effective in *Staphylococcus aureus* infection [89, 90]. There are multitude of results that indicate problem of devising general practices for treatment. There could be the development of conceptual frameworks to follow in administering aggressive and moderate chemotherapy [91], but quantitative systematic analyses are also needed. The challenge was to identify among the diverse potential treatment regimens, that minimized selection for drug-resistance while not compromising patient health [92]. This will go a long way to assist in treating majority of infected people without any side effect.

7. Controlling microbial infection: The best way

Currently, the phenomenon of multi-drug resistance due to indiscriminate administration of high-doses of antibiotics has been the bane of controlling microbial infection. The indiscriminate and inappropriate use of drug in treating

infection has also led to significant toxicity in the infected patients, which present other challenges to tackle. The environment plays a major role in facilitating transmission of several important health care-associated pathogens. These included vancomycin-resistant *enterococci* (VRE), *Clostridium difficile, Acinetobacter spp., methicillin-resistant Staphylococcus aureus* (MRSA) and *norovirus* [93–95]. These pathogens are frequently shed into the environment to contaminate, water and surfaces of any objects for days and increase the risk of infection of humans. In addition, infection occur through vectors of many pathogens, which spread quickly and affect human population.

Together in the environment, microorganisms form complex communities that play critical roles in either maintaining the well-being of their hosts or destroying the host. In order not to allow their survival to the detriment of the existence of the host, they have to be cleared in both the host and the environment. Therefore, several treatment means have been developed to control microbial infections and these have led to the development of antimicrobial drug resistance pathogens. Addressing this challenge, appropriate use of antimicrobials in human medicine is needed. There should be a means of ensuring timely production and communication of critical diagnostic results and standardized drug susceptibility testing reports in accordance with local treatment guidelines [96, 97]. Also, there should be provision of facility-specific cumulative susceptibility reports for bacterial pathogens against antibiotics, daily counseling to clinicians on etiological infection diagnoses and management, and interpretation of test results. Targeted therapy of difficult-to-treat resistant pathogens and complicated infections are very important guidelines in successful treatment of patients. However, some treatment regimens have been developed to be very useful to avoid the development of microbial resistance. These included the use of nanoparticles to destroy the biofilms and also lessen the doses of antibiotics required in treating patients [98]. The development of a recombinant lysis-deficient *Staphylococcus aureus* phage P954, to kill the target cells but not destroy the host cells would alleviate the concern about the use of bacteriophages for therapeutic purposes [99]. These damping the potential immune response, rapid toxin release by the lytic action of phages, and in dose determination difficulty in clinical situations. Phage therapy was currently practiced routinely and successfully in countries such as Poland and Russia [100] and could be developed rapidly to combat the emergence of antibiotic-resistant pathogenic bacteria [101, 102].

Mast cells (MCs) have also been shown to contribute to host–defense responses in certain bacterial infections. Treatment with recombinant IL-6 from engrafted mast cells enhances bacterial killing and resulted in the control of wound infection and normal wound healing [103]. Taken together, host innate immune response will be a potential means in boosting the clearing of microbial organisms.

Generally, public health strategies in controlling infectious diseases needed proper coordination, planning of infection control activities, post-prescription review, and feedback [93, 104, 105]. There should be a team of Clinical Microbiologist and well equipped laboratories with experience staff, working together to inform and improve individual patient care, contribute to outbreak management of infection and provide accurate surveillance data on infectious diseases. This information could be subsequently used in the review of local treatment guidelines, the design and evaluation of national health policies [106].

8. Conclusion

The microbial infection involved the use of many strategies by the pathogens to survive in the host. These have resulted in the development of drug resistance

strains in many pathogens, which persist and continue to be harmful to the host. Many treatment strategies have been failing and making it difficult in controlling diseases. This requires the development of revised scientific means to successfully control infections. Therefore, successful treatment of infections including bacterial and viral infections is the enhancement in both the use of antibiotics (for bacterial infections), antiviral (viral infections) and the host's immune defenses. As a result of the development of drug resistant strains in many treatment cases the enhancement of mostly innate immune response together with the adaptive immune response will go a long way in treating patients without difficulty.

Acknowledgements

The authors would like to thank the staff of the Department of Microbiology and Immunology, School of Medical sciences, University of Cape Coast for their support during the preparation of the manuscript.

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Chapter 3

Role of Kupffer Cells in Systemic Anti-Microbial Defense

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Abstract

The liver has long been recognized as important in digestion. However, the liver's abundance of innate immune cells strongly suggests that it has specific defense mechanisms. A characteristic anatomical feature of the liver is its large blood flow. The blood flowing out from the whole alimentary tract is transported to the liver via the portal vein and distributed to peripheral structures called sinusoids. Kupffer cells, a typical example of resident macrophages, are located in sinusoids and are in continuous contact with various portal blood components. They have vigorous phagocytic activity and eliminate bacteria coming from the gut before they enter systemic circulation. Based on this framework, Kupffer cells were considered a filter for portal blood pathogens. However, recent evidence reveals that they exert crucial functions in systemic host defense against bacterial infection. To defend against various sources of bacterial pathogens, Kupffer cells construct an efficient surveillance system for systemic circulation, cooperating aggressively with other immune cells. They collaborate with non-immune cells such as hepatocytes and platelets to potentiate defense function. In conclusion, Kupffer cells coordinate immune cell activity to efficiently defend against infections, making them crucial players in systemic antibacterial immunity.

Keywords: liver, Kupffer cells, innate immunity, macrophages, bacteria

1. Introduction

The liver is one of the largest organs in the mammalian body and plays an essential role in maintaining health [1, 2]. The hepatic vascular system has a unique and distinct anatomical structure. All veins from the digestive tract unite and form the portal vein. Interestingly, this sizable venous vessel branches into capillaries called sinusoids (indicated by arrows in **Figure 1**) for peripheral microcirculation in the liver. Venous blood from the digestive tract flows into the liver and is processed by hepatocytes before returning to systemic circulation (**Figure 2**). This unique vascular structure of the liver constitutes an ideal environment for innate immune cells to eliminate harmful materials in the blood. Portal blood is filled with beneficial nutrients and unwanted microorganisms ingested along with food. The gastrointestinal tract is also filled with numerous commensal bacteria that form the microbiota. Furthermore, 70% of intravenously injected bacteria accumulate in the liver and are removed therefrom [3]. Thus, bacterial materials in systemic circulation and the portal vein are brought

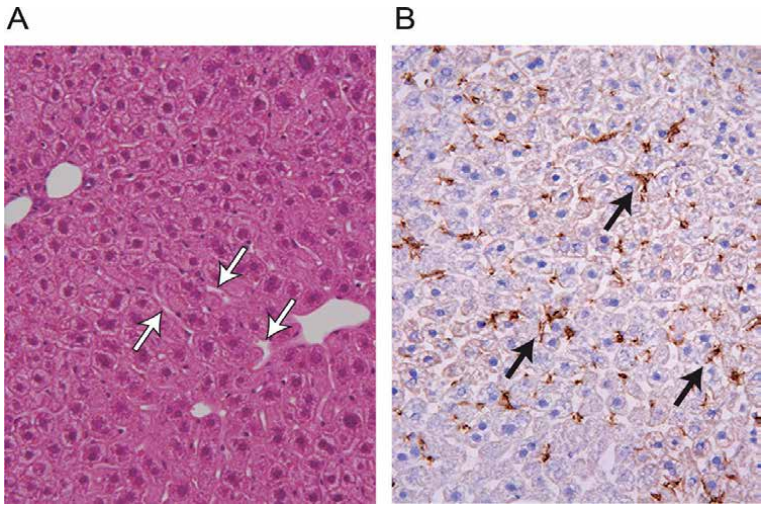

Figure 1.
Microstructure of the liver. (A) Hematoxylin and eosin (HE) staining of the liver (× 400). The portal venous blood and systemic arterial blood are mixed and flow through the sinusoidal space, which is a narrow space for microcirculation between numerous hepatocytes (white arrows). (B) Immunohistochemical staining of the mouse liver (× 400). The primary antibody against F4/80 antigen, which is a specific marker for the macrophage in mice, was reacted and followed by horseradish peroxidase staining (brown area). Counterstaining was performed by hematoxylin to distinguish hepatocytes (blue area). The sinusoidal space is lined with a large number of F4/80-positive Kupffer cells (black arrows). Overall, the blood stream passes through two types of filters, nutritional processing and immunological surveillance.

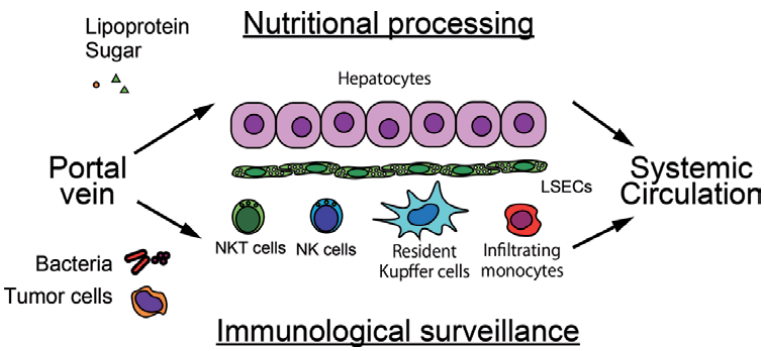

Figure 2.
The two kinds of filtering systems in the liver. One involves the nutritional processing of absorbed sugars and lipids, which is supported by hepatocytes. The other involves immunological surveillance of external pathogens, such as bacteria and tumor cells, through a unique innate immune cell network. These two cell types are separated by liver sinusoidal endothelial cells (LSECs).

to the liver and activate innate immune cells, which are essential for eliminating pathogenic organisms in the host. The narrow space of the sinusoids and slow blood flow form an ideal environment for eliminating pathogenic microorganisms entering the liver. Recently, many researchers have examined the liver as an innate immune organ based on anatomical and immunological viewpoints [4, 5].

2. The liver demonstrates the structure required for antibacterial responses

The liver contains unique innate immune cells, including natural killer (NK) cells, natural killer T (NKT) cells, and Kupffer cells [1]. These innate immune cells

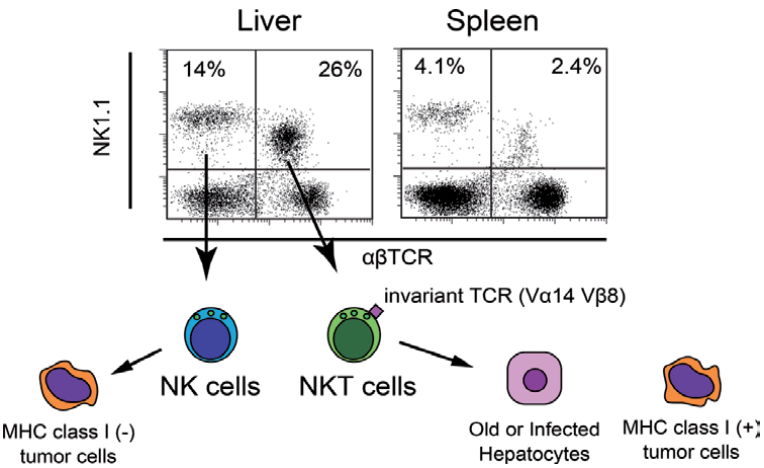

Figure 3.
The distinct composition of T cells in the liver. Liver and spleen lymphocytes were isolated from C57BL/6 mice and subjected to flow cytometry analysis. Isolated cells were developed into two-dimensional histograms with the αβ T-cell receptor (TCR) and NK1.1 antigen. In the liver, double-positive natural killer T (NKT) cells, and single-positive natural killer (NK) cells comprised a larger population than in the spleen. NK cells exert strong anti-tumor cytotoxicity against major histocompatibility complex (MHC) class I negative tumors. NKT cells can induce apoptosis in old or infected hepatocytes and MHC class I-positive tumor cells.

carry out essential bilateral immunological functions, such as antibacterial and anti-tumor immunity. Kupffer cells are the most well-known tissue-resident macrophages and are pivotal effectors of antibacterial immunity [6]. They are characterized by vigorous phagocytic activity [7]. Most Kupffer cells exist in the zone 2 region of the sinusoids, where the blood flow is the slowest [8] (**Figure 1B**). They express scavenger receptors and constantly engulf exogenous materials, such as bacteria. NKT cells comprise approximately 25% of the hepatic lymphocytes, which is a high percentage compared to other organs [1] (**Figure 3**). Typical NKT cells have an invariant T-cell receptor (TCR). In contrast to conventional T cells, their TCR shows much less variation; approximately 90% of them express Vα14-Jα18 in mice, which may recognize antigen "patterns" rather than specific antigen structures. The invariant TCR of NKT cells is reported to recognize a synthetic glycolipid, α-galactosylceramide, or some bacterial structures [9]. However, the natural ligands of NKT cells remain to be elucidated. Along with NK cells, the essential function of NKT cells is now considered to be anti-tumor response [10–12]. In contrast, macrophage populations are essential cellular factors for bacterial defense in the liver [13].

3. Two distinct macrophage subsets in the liver

Each organ has a specific macrophage subset. Generally, bone marrow-derived monocytes infiltrate tissues and differentiate into tissue-resident macrophages [14]. The constitution of macrophages in the liver is more complex. The liver tissue-resident macrophages or Kupffer cells are derived from yolk sac-originated progenitor cells and are self-renewed in the liver, independent of the bone marrow [15]. In contrast, bone marrow-derived infiltrating monocytes coexist in the sinusoidal space and play essential roles in inflammatory reactions (**Figure 4**) [16, 17] . They are positive for the lymphocyte antigen 6 complex (Ly6C), which is a typical marker for bone marrow-derived immune cells. Interestingly, these two macrophage subsets possess various differing features. Kupffer cells exhibit vigorous phagocytic activity and longer self-renewal time. They disappear in response to clodronate liposome

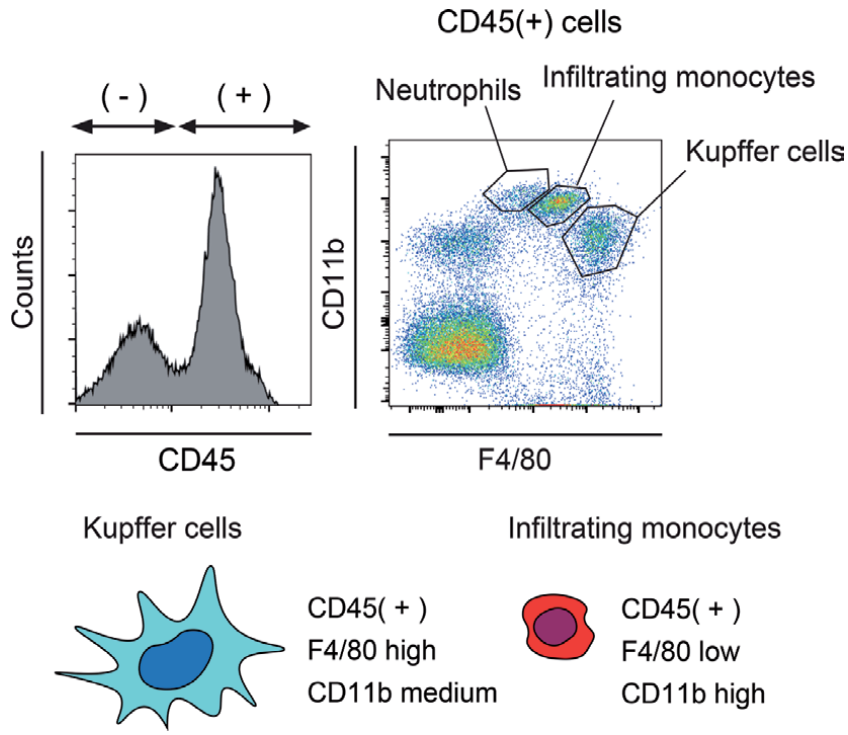

Figure 4.
The composition of macrophages and neutrophils in the liver. Non-parenchymal cells were isolated from the mouse liver and examined by flow cytometry to analyze macrophage composition. Immune cells were selected with the CD45 antigen, and a two-dimensional histogram was plotted against F4/80 and CD11b antigens. F4/80 high and CD11b medium cells were Kupffer cells; F4/80 low and CD11b high cells were infiltrated monocytes; neutrophils comprised the CD11b highest population; eosinophils, which are also F4/80 positive, were excluded using the Siglec-F antigen.

treatment [18, 19], which induces apoptosis of macrophages after phagocytosis. Their proliferation is independent of bone marrow, and their longer turnover cycle confers resistance to radiation exposure [20, 21]. In contrast, infiltrating monocytes potently secrete inflammatory cytokines and accelerate inflammation; they are less phagocytic and are rapidly supplied from the bone marrow [16, 22]. Furthermore, they are resistant to clodronate liposome treatment and are susceptible to radiation exposure [23]. These two lineages of macrophages cooperate to eliminate exogenous pathogens from the bloodstream.

4. Vigorous phagocytic activity of Kupffer cells

Kupffer cells are characterized by their vigorous phagocytic activity. They can engulf fluorescein isothiocyanate (FITC)-labeled *Escherichia coli* (FITC-*E. coli*) more efficiently than the infiltrating monocytes (**Figure 5**). The immediate initial response was also a remarkable feature. Kupffer cells phagocytose FITC-*E. coli* immediately after *in vivo* administration, which was much faster than that by infiltrating monocytes (**Figure 6**). This feature suggests they have a sophisticated ability to distinguish foreign pathogens, such as bacteria. From this viewpoint, it is natural to recognize them as key players in eliminating systemic bacterial loads, such as in severe sepsis. Notably, they can actively phagocytose both gram-negative and positive bacteria [23]. In 1959, Benacerraf et al. reported that the blood clearance rate of gram-positive *Staphylococcus aureus* (*S. aureus*) was much faster than that

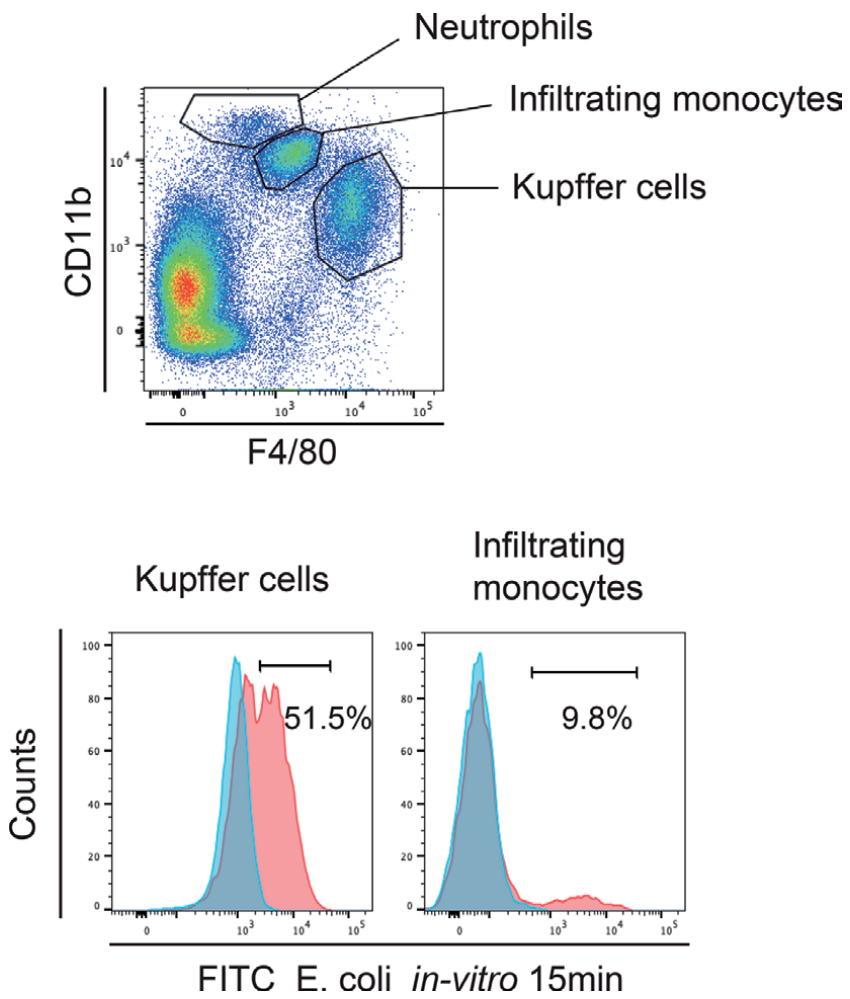

Figure 5.
Evaluation of phagocytosis by liver immune cells in vitro. Liver immune cells were isolated and incubated with FITC-labeled Escherichia coli (E. coli). After 15 minutes (min) of incubation, the cells were collected and analyzed using flow cytometry. Approximately half of the Kupffer cells engulfed the bacteria (red area), which is much more efficient than monocytes. The blue area represents the sample with no bacteria and is set as a negative control. Kupffer cells showed strong auto-fluorescence, and the blue area was shifted to the positive side.

of gram-negative *E. coli*, and almost all of them were trapped in the liver [3]. They also suggested that opsonization by immunoglobulin was not necessary because the clearance rate was very rapid. This report strongly suggests that Kupffer cells play a significant role in the clearance of gram-positive cocci in the blood stream. *S. aureus* usually invades the bloodstream from inflammatory lesions in the skin, oral cavity, and respiratory system. As Kupffer cells actively phagocytose this type of bacteria, it is evident that they play an essential role in protecting against pathogens derived from systemic circulation, not only from the portal vein. One of the characteristic genes of Kupffer cells is the complement receptor of the immunoglobulin superfamily (CRIg) [24]. CRIg directly binds to gram-positive bacteria through lipoteichoic acid, independent of complement [25]. This process is essential for effectively eliminating gram-positive bacteria from the bloodstream in the liver. Consistently, after elimination of Kupffer cells by treatment with clodronate liposomes, the survival rate after intravenous challenge with live *S. aureus* was significantly decreased [23] (**Figure 7A**). The Kupffer cell elimination blunts the liver's clearance ability and renders the mice more susceptible to the *S. aureus* (**Figure 7BC**).

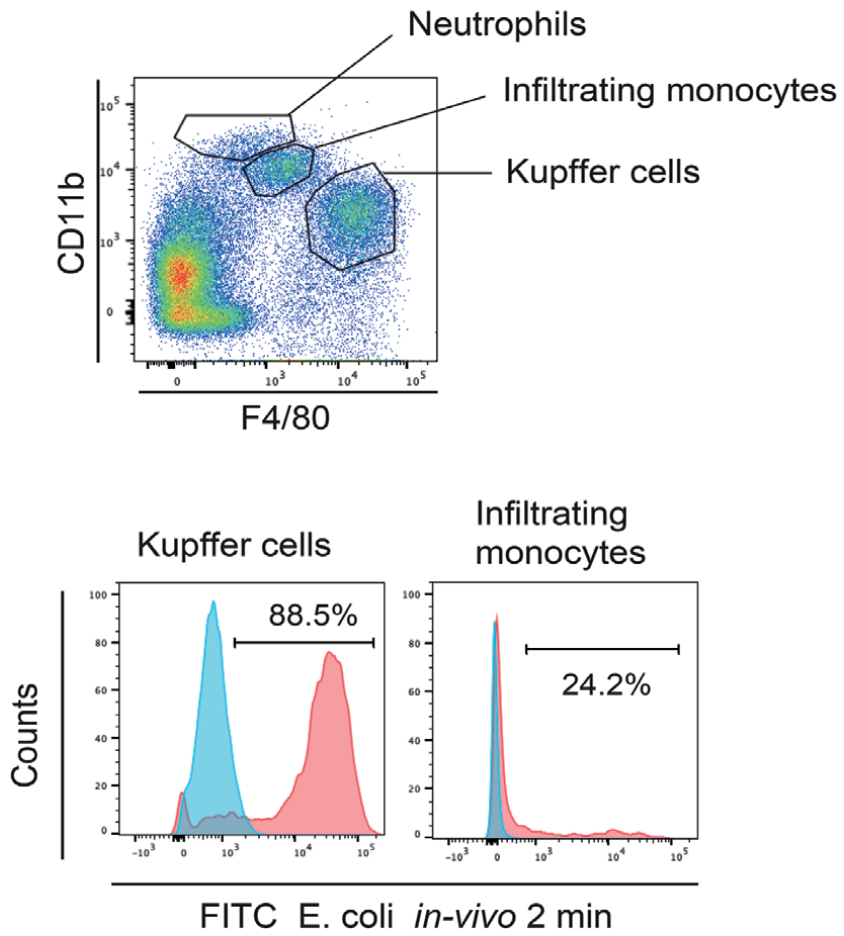

Figure 6.
Evaluation of phagocytosis by liver immune cells in vivo. Mice were intravenously injected with FITC-labeled E. coli via the tail vein. Liver immune cells were isolated 2 min after injection and analyzed by flow cytometry. The blue area is the sample from the mice injected with unlabeled control bacteria, set as a negative control. Approximately 90% of Kupffer cells engulf or attach the bacteria after only 2 min (red area), demonstrating their rapid and vigorous phagocytic activity.

5. Activation of Kupffer cells by infiltrated monocytes

A substantial number of monocytes exist in the liver, as well as in other organs. These can be isolated even after intense perfusion from the portal vein, and their numbers are markedly increased by systemic inflammation or experimental hepatitis [26]. These phenomena indicate that they are not aberrant bystander cells in the liver. They are recruited from the bone marrow, actively attach to the sinusoidal space, and play a specific role in the hepatic immune mechanism. Their definition and nomenclature are still controversial; some investigators call them infiltrating monocytes, whereas others refer to them as monocyte-derived macrophages. Both M1-like proinflammatory and M2-like immunomodulatory populations were present in this subset. These complexities have stimulated much discussion and controversy. Although their strict definition still requires future study, some of their primary functions are already known [21, 27]. Regarding immune reactions, Ly6C$^+$ monocytes produce proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-12

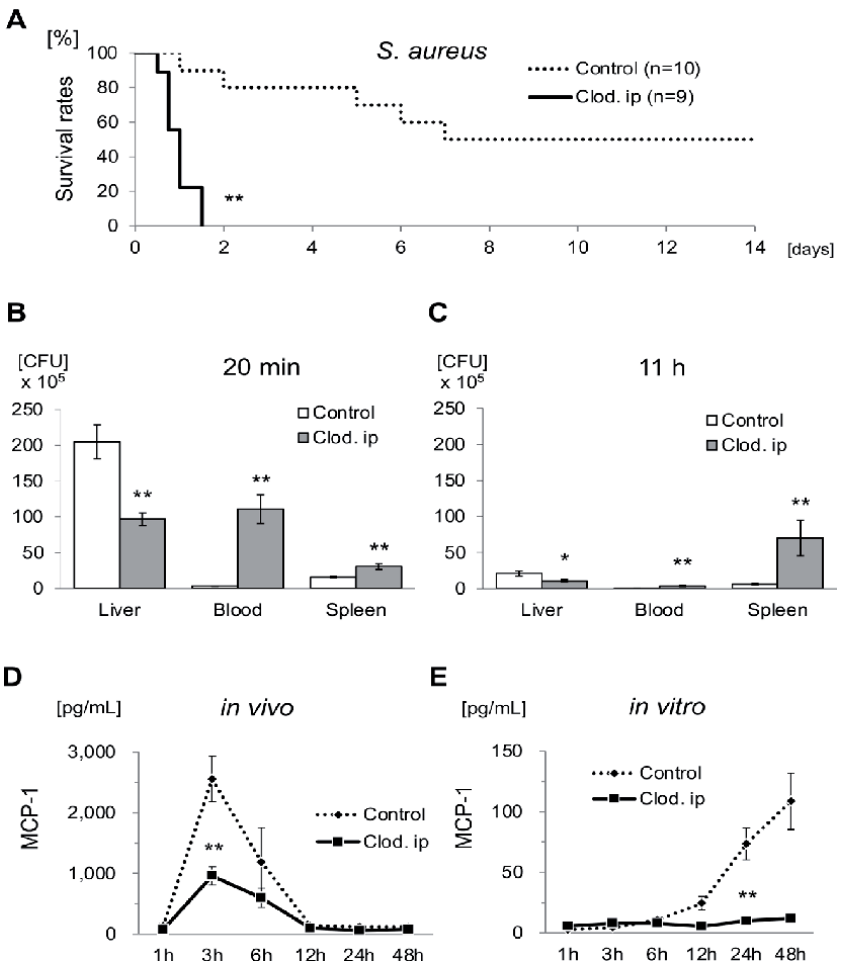

Figure 7.
*Clodronate pretreatment made mice susceptible to Staphylococcus aureus (S. aureus) infection. (A) In clodronate liposome-pretreated mice, the survival rate of mice infected with S. aureus was significantly decreased (solid line) compared to control mice (dotted line). (B) The number of bacteria trapped in the liver was decreased in clodronate treated mice (gray columns) compared to control mice (white columns). The un-trapped bacteria were remaining in the blood and the spleen. After 20 minutes of S. aureus injection, each organ was collected, homogenized and colony forming units (CFUs) were analyzed. (C) After 11 hours, the certain number of bacteria remaining in the spleen in clodronate-pretreated mice. (D) The MCP-1 level in sera after injection of S. aureus significantly decreased in clodronate-pretreated mice (solid line) compared to control mice (dotted line). (E) The MCP-1 production of liver immune cells by incubation with S. aureus was inhibited in clodronate-pretreated mice (solid line) compared to control (dotted line), which means Kupffer cells are the main source of this chemokine. *P < 0.01, **P < 0.05 versus control in unpaired student t test [23].*

(IL-12) [22]. In some experimental hepatitis models, FasL expressed by these cells acts as a final effector to injure hepatocytes that express Fas [26], inducing Fas–FasL-dependent apoptosis [28, 29]. In bacterial defense, Kupffer cells engulf bacteria and produce chemokines such as monocyte chemoattractant protein-1 (MCP-1) (**Figure 7DE**) and recruit these monocytes into the sinusoidal space. Such recruited monocytes produce inflammatory cytokines such as TNF and facilitate Kupffer cell's antibacterial activity [23]. If this pathway is blocked using a recombinant TNF antibody, reactive oxide production from Kupffer cells is inhibited, and their bactericidal activity is reduced [30, 31]. This cell population is thus essential for effective elimination of bacteria by Kupffer

cells, and the combination of these two macrophage populations is crucial for an effective immune response against bacteria.

6. Regulation of Kupffer cell functions by C-reactive protein (CRP)

CRP is an acute-phase protein produced by hepatocytes during inflammation. The serum level of this protein is recognized as a marker for evaluating inflammation severity. The sensitivity and specificity of serum CRP levels are high enough to detect even minor inflammation in the body. According to recent research, this acute-phase protein is a clinical marker as well as an important protein that drives macrophage activity into a preferable and reasonable state [32, 33]. Pretreatment with synthetic CRP improved survival after intravenous bacterial challenge (**Figure 8**). The mechanism underlying this reaction is the increased phagocytic activity of Kupffer cells and the suppression of excessive inflammatory cytokines from activated monocytes. Overall, treatment with synthetic CRP drives the immune cell system to a preferable state and improves survival in bacterial infections. In addition to the beneficial effect of synthetic CRP, the natural form of CRP reportedly has various means of modulating immune functions [34]. Although the primary functions of hepatocytes is commonly accepted to be involved in processing nutrition, it is suggested that hepatocytes have immunomodulatory functions, based on the fact that they are involved in the production of complement proteins and acute phase proteins such as CRP. This aspect of hepatocytes is consistent with the theory that the liver is a crucial organ in systemic antibacterial immunity.

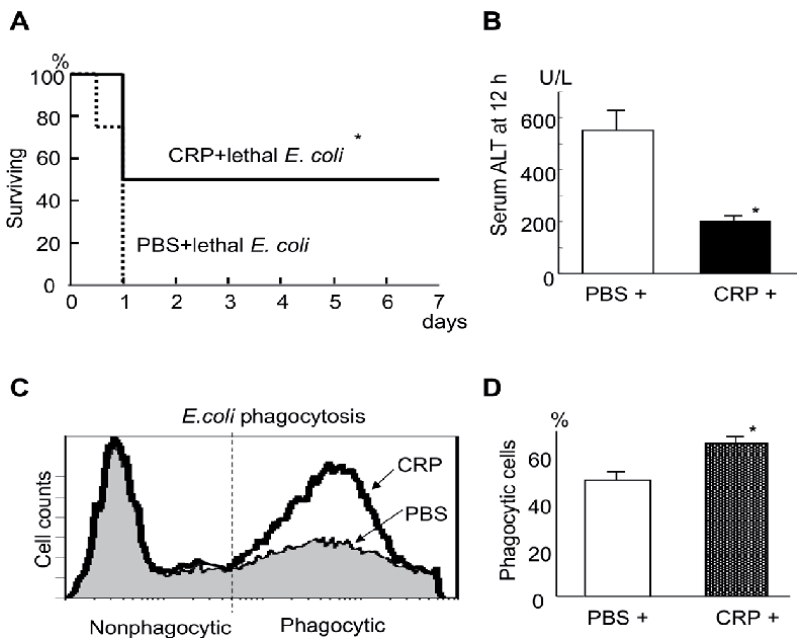

Figure 8.
*Synthetic CRP improved the survival rate of lethal E. coli infection in mice. (A) C57BL/6 mice were pretreated with synthetic CRP (C-reactive protein) or phosphate buffered saline (PBS) and were challenged intravenously with a lethal dose of E. coli. Survival rate was improved by synthetic CRP. (B) Liver dysfunction after 12 hours (h) of E. coli injection was ameliorated in CRP treated mice (black column). (C) CRP- or PBS-pretreated mice (1 hour before) were injected intravenously with FITC labeled E. coli. Liver immune cells were isolated after 20 minutes and analyzed with flow cytometry. Kupffer cells were gated, and phagocytosis of FITC-E. coli was demonstrated. (D) The proportion of phagocytosing Kupffer cells is increased in CRP treated mice. *P < 0.01 versus other groups in unpaired student t test [32].*

7. Relationship with neutrophils

The liver is highly responsive to invasion by external antigens from various origins [5]. Kupffer cell show the ability to engulf microorganisms. However, they have one serious disadvantage. Namely, their self-renewal speed is slower than that of other immune cells. For instance, after injection of clodronate liposomes, which can eliminate almost all Kupffer cells, at least two weeks are required to restore Kupffer cell numbers [6]. Upon exposure to an excessive number of bacteria, their phagocytic ability reaches its limit by repeated phagocytosis, and they easily undergo apoptosis and disappear from the sinusoidal space [35]. Their ability to attract other immune cells with chemokines seems to be a compensatory reaction to overcome this adverse effect. They recruit monocytes and neutrophils into the sinusoidal space to support the clearance of an excess number of bacteria. A previous report described that Kupffer cells attach bacteria on their cell surface and that the main effectors phagocytosing bacteria are neutrophils [36]. Consistent with this report, Kubes *et al.* reported that neutrophils clear the bacteria by cooperating with Kupffer cells in the presence of platelets [37]. Neutrophils phagocytose bacteria and form neutrophil extracellular traps (NETs) in the sinusoidal space to facilitate bacterial clearance.

8. Relationship with platelets

C-type lectin 2 (CLEC2) is a characteristic marker of Kupffer cells [38]. All Kupffer cells showed high expression of this antigen, which has been recognized as a marker for their identification in flow cytometric analyses. CLEC2 is a receptor for platelets, and it may be unclear why this antigen is highly expressed in Kupffer cells. The primary function of platelets is hemostasis, which is profoundly different to the immunological defense mechanism. However, platelets also express various immunological markers, such as toll-like receptors, and contribute to immunological functions [39, 40]. The specific role of platelets in liver immune reactions was previously reported in 1992 [41]. In this report, platelets in the blood were found to migrate rapidly to the liver after systemic bacterial antigen administration. The mechanism underlying this reaction was reported in 2013 [42]. Under normal conditions, platelets maintain continuous contact with Kupffer cells. However, in systemic gram-positive bacterial infection, Kupffer cells bind bacteria transported via the bloodstream, attach them to their cell surface, and form aggregates with platelets. These aggregated complexes facilitate NET development by neutrophils in the sinusoidal space. Along with the vigorous phagocytosis by Kupffer cells, this reaction also contributes significantly to the clearance of harmful bacteria from blood [43]. Interestingly, this reaction is augmented by complement component C3, which is produced by hepatocytes [42]. Thus, this reaction exemplifies a sophisticated collaboration network of Kupffer cells with platelets, neutrophils, and even hepatocytes in the systemic bacterial defense mechanism.

9. Conclusion: Kupffer cells are crucial immune cells for systemic antibacterial defense

The remarkable immunological abilities of Kupffer cells, such as phagocytosis, reactive oxygen species production, and antigen presentation, strongly suggest their enormous contribution to immunological responses. Based on the vascular

architecture of the liver, Kupffer cells have been recognized as playing pivotal roles in eliminating portal vein-derived pathogens from the intestinal tract. However, increasing evidence indicates that they are crucial effectors in systemic defense mechanisms against bacteria, cooperating with other immune cells such as monocytes, neutrophils, and even non-immune such as hepatocytes, and platelets. From this viewpoint, Kupffer cells are phagocytic scavengers and conductors orchestrating the effective elimination of blood-borne bacteria. Thus, Kupffer cells play a crucial role in systemic antibacterial defenses.

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Chapter 4

The Role of the Aryl Hydrocarbon Receptor (AhR) in the Immune Response against Microbial Infections

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Abstract

Aryl hydrocarbon receptor (AhR), an important nuclear receptor, regulates the cellular response to environmental stressors. It is well known for its critical functions in toxicology, but is currently considered an essential regulator of diseases, with specific modulatory effects on immune, antimicrobial and inflammatory responses. The present chapter discusses AhR's function and mechanism in the immune response against microbial infections.

Keywords: aryl hydrocarbon receptor (AhR), functional mechanism, antimicrobial, immunity, gut immunity

1. Introduction

The ligand-activated transcription factor aryl hydrocarbon receptor (AhR) is structurally similar to other members of Pern-Arnt-Sim (PAS) superfamily [1, 2], which consists of a conserved signaling network that regulates signal exchange between host and environment [3, 4]. It was originally found to play a role in regulating the reactions of exogenous chemicals such as 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). However, AhR has been recently recognized as an essential regulator of host-pathogen interactions [5–9], especially affecting immunity, inflammatory response and antibacterial activity [5, 9–15]. The current chapter focuses on AhR's function in regulating immunity, inflammatory response and antibacterial activity.

2. Mechanism of AhR action

As a highly conserved nuclear receptor [10], AhR can regulate gene expression after binding to a ligand. AhR binds to its co-chaperones and maintains cytoplasmic localization [16, 17]. Ligand binding by AhR results in its release by co-chaperones and translocation into the nucleus, where it forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) [18, 19]. Via binding to the genomic DNA—usually interacting with AhR response elements (AhREs, 5'-GCGTG-3′) [20, 21], also referred to as dioxin (DREs) or xenobiotic (XREs) response elements [9, 10],

the AhR-ARNT heterodimer regulates multiple target genes such as Cytochrome P450 Family 1 Subfamily A Member 1 (CYP1A1), CYP1A2, CYP1B1, TCDD Inducible Poly (ADP-Ribose) Polymerase (TIP ARP), and aryl hydrocarbon receptor repressor (AhRR), which can inhibit AhR via a negative feedback circuit [22]. Target gene regulation is considered to be ligand dependent [21].

As a highly heterogeneous nuclear receptor, AhR binds to many ligands, including exogenous synthetic aromatic hydrocarbons [10, 23], exogenous natural chemicals [5, 6, 10, 14, 24] and endogenous ligands [25–29]. Tryptophan, an essential amino acid in humans, constitutes the precursor of many important components in the human body. Interestingly, the tryptophan (TRP) pathway has a critical function in immune and inflammatory responses through providing many ligands for AhR. In addition, AhR controls the expression and activation of tryptophan 2,3-dioxygenase (TDO2), indoleamine 2,3-dioxygenase (IDO), kynureninase (KYNU) and kynurenine 3-monooxygenase (KMO). The aforementioned enzymes catalyze the synthesis of kynurenine (KYN), which is a product of TRP metabolism, thus enabling feedback inhibition because KYN and AhR are agonists [30, 31].

3. AhR expression modulation

The interactions of AhR and its ligands, including polycyclic aromatic hydrocarbons (PAHs), can be used as a cytoplasmic signal sensor. The conformation of AhR changes, and it is transferred from the cytoplasm to the nucleus. The high-affinity ligand TCDD can exert toxic effects by binding with and activating AhR [32, 33]. Structural analysis of AhR revealed three domains: 1) The amino-terminal DNA binding domain (DBD) comprises the basic helix–loop–helix (bHLH) region and the nuclear localization signal (NLS); 2) The central PAS region encompasses two degenerate repeats; 3) The carboxy-terminal region features the transactivation domain (TAD) [34]. In addition, phylogenetic data showed that AhR constitutes an ancient protein whose functional orthologues are found in reptiles, amphibians, birds and mammals. However, there are many structural differences between human and murine AhR genes. Sequence analysis revealed approximately 85% structural similarity in the amino-terminal sequence, while the C-terminal region shows a low homology. The TAD or N-terminal domain is the least conservative [34]. The C-terminal domain is a highly unstructured sequence containing a transcriptionally active region and contributes to receptor transformation [35, 36].

AhR, heat shock protein 90 and X-associated protein 2 form multiple protein complexes in the cytoplasm. In the presence of ligands or agonists, AhR complexes undergo nuclear translocation and form heterodimers with ARNT. With a core sequence of 5′-GCGTG-3′, the AhR/ARNT complex interacts with DREs in the proximal site of promoters of target genes. Both AhR and ARNT recruit additional transcription co-activators for gene regulation, e.g., CYP and AhRR. Once transferred into the nucleus, AhR undergoes proteasome-induced degradation [37]. AhR function is modulated and weakened by AhRR, another member of the PAS family. After AhR activation, the level of AhRR increases rapidly [38]. Meanwhile, AhRR has a transcriptional repressor domain and can dimerize with ARNT even without an agonist, to fulfill its function [39].

4. AhR response to bacterial pathogens

It is known that AhR has a critical function in controlling responses to a variety of microbial pathogens. For example, it is required to effectively clear the

Gram-positive pathogenic bacteria *Listeria monocytogenes* (LM). In mice, AhR inhibits LM by inducing ROS production via upregulation of the anti-inflammatory cytokine IL-10 and macrophage apoptosis inhibitor, resulting in suppressed macrophage apoptosis, reduced amounts of pro-inflammatory cytokines (e.g., Interleukin 6 (IL-6) and Tumor Necrosis Factor alpha (TNF-α)), and decreased the nuclear factor kappaB (NF-κB) activation. In addition, AhR ligands can enhance the response of AhR WT mice to LM, but not of $AhR^{-/-}$mice [27].

When inoculated with log-phase LM intravenously, AhR deficient C57BL/6 J mice ($AhR^{-/-}$) showed higher susceptibility compared with AhR heterozygous ($AhR^{+/-}$) littermates. In comparison with $AhR^{+/-}$ animals, $AhR^{-/-}$ counterparts showed more colony forming units (CFUs) of LM in the spleen and liver, and more pronounced alterations in liver histopathology. Serum monocyte chemotactic protein 1 (MCP-1), IL-6, TNF-α and Interferon γ (IFN-γ) amounts were similar in $AhR^{-/-}$and $AhR^{+/-}$mice infected with LM. Elevated IL-12 and IL-10 amounts were detected in $AhR^{-/-}$mice infected with LM. In terms of capacity of uptake and inhibition of intracellular growth of LM, $AhR^{+/-}$and $AhR^{-/-}$macrophages were comparable *in vitro*. In addition, T cell-dependent response was similar in $AhR^{-/-}$and $AhR^{+/-}$mice, as determined by intracellularly labelling cluster of differentiation 4 and 8 ($CD4^+$ and $CD8^+$) splenocytes for IFN-γ and TNF-α. $AhR^{-/-}$and $AhR^{+/-}$mice with prior infection showed increased resistance to re-infection by LM. The above evidence suggests that AhR is necessary to build an effective resistance, but not required for adaptive immune reactions following LM infection [40].

Streptococcus pneumonia, a common respiratory pathogen, represents a major cause of morbidity and death in humans, especially the elderly and children. The immune response after *S. pneumoniae* infection begins quickly in the lung, and the innate immune response can contain bacterial colonization in the ideal situation. Death, and bacterial load, cytokine/chemokine amounts, and immune cell infiltration in the lung have been assessed at different times in TCDD treated mice after *S. pneumoniae* infection. The survival rate of mice administered TCDD was significantly increased, while bacterial load in the lung was reduced. However, intriguingly, no evidence suggested that the protective effect was caused by increased inflammatory response. In fact, neutrophil amounts and inflammatory chemokine/cytokine levels in TCDD treated mice were lower than those of control animals. These findings suggest that AhR induction does not protect the animals by immune modulation, but likely by directly affecting lung cells upon infection [41].

Pseudomonas plecoglossicida represents the bacterial pathogen of fish visceral white spot disease with temperature dependent virulence [42]. AhR is also required for resistance to *P. plecoglossicida*. It was shown that *ahr1a*, *ahr1b*, *ahr2* and *cyp1a* amounts in various organs of *Danio rerio* and *Epinephelus coioides* infected with *P. plecoglossicida* have similar trends. It should be noted that the intestine, liver, heart and spleen are the most affected organs, while *ahr2* specifically shows a sharp increase in the spleen. After *P. plecoglossicida* infection, *ahr1a* amounts in macrophages are markedly reduced, while *ahr1b*, *ahr2* and *cyp1a* are overtly upregulated. The cell viability and immune escape rates of *P. plecoglossicida* were significantly increased in macrophages with *ahr1b* and *ahr2* knockdown. In conclusion, *ahr1a*, *ahr1b*, *ahr2* and *cyp1a* are involved in immune reactions to *P. plecoglossicida* in various fish organs, while *ahr1b* and *ahr2* might play a key role in splenic and macrophage immune reactions [43].

Huang et al. described the first pathogenic *Aeromonas salmonicida* (SRW-OG1) obtained from the warm water fish *E. coioides*, and studied AhR's role in the immune response to SRW-OG1 infection [44]. They found that AhR is induced by unknown ligands in the intestine, spleen and macrophages. At the same time, *ahr1a* and *ahr1b* amounts were markedly elevated in the intestine, spleen and macrophages,

while *ahr2* only showed an increase in the intestine, suggesting *ahr2* may contribute less to immune reactions compared with *ahr1a* and *ahr1b*. In SRW-OG1 infected *E. coioides*, major genes contributing to bacterial recognition, macrophage inflammatory response and gut immunity were overtly upregulated. However, decreased ROS amounts and the downregulation of other associated genes were equally detected, which indicated that SRW-OG1 could prevent ROS production by macrophages through its virulence mechanism. In addition, repression of AhR with an inhibitor or by gene silencing rescued the increases of *IL-1 β* and *IL-8* associated with SRW-OG1 infection, clearly demonstrating that induction of *E. coioides* macrophages by *IL-1 β* and *IL-8* is controlled by AhR. Nevertheless, AhR exerted no effects on bactericidal permeability-increasing protein/lipopolysaccharide-binding protein (BPI/LBP), reactive oxygen species (ROS) biosynthesis and associated genes. Compared with wild-type macrophages, survival and immune escape rates after SRW-OG1 infection were significantly increased in *ahr1a*/*ahr1b*-knockdown and 3′, 4’-DMF treated macrophages. Taken together, *ahr1a* and *ahr1b* are necessary for the immune response to SRW-OG1 [44].

Lipopolysaccharide (LPS) stimulation is often utilized to model Gram-negative bacteria-induced sepsis for assessing AhR's functions in infection resistance and septic shock regulation. AhR and TDO2 are required for survival after the initial exposure to LPS [14, 20], while subsequent exposures are dependent on AhR and IDO1/2. LPS up-regulates TDO2 and IDO1/2, the rate-limiting enzymes of TRP transformation into KYN, and further induces AhR, thus downregulating pro-inflammatory cytokines and regulating long-term systemic inflammation [20]. In addition, compared with AhR wild type mice or immune cells, LPS challenged $AhR^{-/-}$ mice or immune cells produce higher concentrations of pro-inflammatory cytokines, including IL-1 β, IL-6, IL-18, IL-12, TNF-α and IFN-γ, as well as NLR Family Pyrin Domain Containing 3 (NLRP3) that regulates multiple pro-inflammatory cytokines. The AhR agonists 3-methylcholine (3-Mc), 6-Formylindolo[3,2-b] carbazole (FICZ), KYN and TCDD could protect AhR WT mice, but conferred no protection to $AhR^{-/-}$ animals, from extremely high amounts of pro-inflammatory cytokines and septic shock [45]. Thus, the immune response to bacterial pathogens requires AhR, and the underlying mechanisms are vital in identifying novel therapeutic agents to combat bacterial pathogens.

5. AhR response to viral pathogens

AhR is also associated with response to viral pathogens. For example, herpes simplex virus (HSV)-associated eye infection can lead to chronic immune-inflammatory response, causing blindness. However, in a mouse model, a single dose of TCDD could alleviate herpetic keratitis lesions, reduce viral load and decrease pro-inflammatory cytokine levels. However, similar effects were not obtained with FICZ, thus indicating a difference between both AhR ligands [46]. Therefore, response to viral pathogens requires AhR, and nontoxic AhR agonists could be used in the treatment of HSV-induced eye infections.

In influenza virus infection, activation of AhR doubles the number of neutrophils in the airway and interstitium of the lung, which reduces the survival rate from an otherwise sub-lethal infection [47, 48]. Interestingly, no increase in neutrophil inflammation or decreased survival was observed in AhR deficient mice treated with TCDD and influenza virus [37]. Innate immune reactions, including excessive pulmonary neutrophilia, can lead to severer pathological conditions and poor clinical outcomes after influenza virus infection [49–51]. Meanwhile, epidemiological reports have shown that exposure to environmental AhR ligands is associated with

elevated respiratory tract infection, pulmonary congestion and exacerbation of inflammatory lung disease [52–54]. Therefore, there is parallel evidence in rodent animal models and humans that AhR regulates neutrophil inflow during infection. Overall, these data suggest that AhR regulates a new pathway to regulate neutrophil migration during influenza virus infection. A possible new target gene of AhR is inducible nitric oxide synthase (iNOS). Meanwhile, activation of AhR can increase the expression of iNOS in the mouse lung upon infection with influenza virus [55].

6. AhR response to parasitic pathogens

The immune response to parasites also requires AhR. For example, immune response to *Toxoplasma gondii*, a pathogenic parasite causing toxoplasmosis, requires increased AhR-dependent production of IL-10. Indeed, $AhR^{-/-}$ mice have reduced response to *T. gondii* and a less pronounced IL-10 increase [56].

After intraperitoneal infection with *T. gondii*, the death rate of $AhR^{-/-}$ mice was significantly higher than that of WT mice. Moreover, $AhR^{-/-}$ mice showed greater liver injury, and higher levels of NO, IgE and TNF-α, but lower IL-10 secretion in the serum. Interestingly, fewer cysts were found in the brain. The increased mortality was related to reduced IL-10, 5-LOX and GATA-3 expression levels, but increased IFN-γ expression in the spleen. In addition, $AhR^{-/-}$ mice had increased IL-12 and IFN-γ amounts, but decreased TLR2 levels compared with wild-type mice in peritoneal exudate cells. These findings suggest that AhR is vital for limiting inflammation during toxoplasmosis [57].

Therefore, AhR is necessary for parasitic pathogen response. This provides information on a response pathway and can be used to design new treatments.

7. AhR and the intestinal microbiota

AhR is found at high levels in the epithelial barrier [58], and the intestinal barrier of $AhR^{-/-}$mice is inadequate, suggesting AhR might be important in maintaining or generating a healthy intestinal barrier [19]. In addition, low levels of AhR and AhR's target genes are found in sterile mice [9], and AhR is needed for maintaining the $ROR^{\gamma t+}$ innate lymphoblastoid cell (ILC) balance in the intestine [18]. In addition, the TRP metabolizing indole biosynthesized by select bacterial components of the intestinal microbiota is an AhR ligand [59, 60]. Diet without indole or antibiotic treatment can lead to the differentiation of mononuclear phagocytes, dependent on AhR, into dendritic cells (DCs) [48], which are more susceptible to gut pathogens in mice [17]. Overall, the above findings suggest AhR might be important in host gut-microbiota interactions.

AhR also plays a role in the reciprocal relationship among intestinal bacteria, bacterial metabolites and the intestinal immune system. AhR-deficient $ROR\gamma t^+$ ILCs (the main producers of gut IL-22) with lower IL-22 amounts make mice easily die upon *Citrobacter rodentium* infection. It was pointed out that treatment with FICZ markedly enhances $ROR\gamma t^+$ ILC accumulation in $AhR^{+/-}$ and $AhR^{+/+}$ mice, but not in $AhR^{-/-}$ animals [61]. Lactobacillus species (nonpathogenic intestinal bacteria) are capable of producing AhR ligands, including indole-3-aldehydes, from tryptophan in the gut, thus enhancing the production of AhR dependent IL-22 [62]. Indole-3-aldehydes induces AhR-associated transcription, but exclusively at elevated concentrations, indicating its low affinity. However, indole-3-acetaldehyde (a product of indole-3-aldehydes) produces the high-affinity ligand FICZ [63], which may be related to the effect reported by Zelante et al. IL-22 affects epithelial cells and

causes them to produce antimicrobial peptides, such as type III Reg (regenerating gene product) gamma (RegIIIg), and to stimulate tissue regeneration. Meanwhile, symbiotic bacteria may outperform bacterial pathogens and inhibit *Candida albicans* colonization [51]. Similar to keratinocyte and skin immune cell levels, AhR amounts are high in IECs and intestinal immune system cells [64].

In AhR-null mice, the number of intraepithelial lymphocytes (IELs) in the small intestine is significantly reduced [6, 64, 65], which is related to lower IL-22 amounts, and therefore to downregulated ileal antimicrobial peptides, including RegIIIb and RegIIIg. The microbial loads of the small and large intestines are also elevated. Loss of IELs is cell-intrinsic since AhR-deficient bone marrow cells do not reconstruct the gut in $Rag^{-/-}$ mice [51]. Over time after birth, intestinal Group 3 Innate Lymphoid Cells (ILC3s) [66], ILC22 and CD32NKp46^{+} lymphoid tissue inducer cells are lost in AhR-deficient mice. Similarly, ILC3's inability to multiply in AhR-deficient mice constitutes an intrinsic function since AhR is required for the transcription of the cell-specific proliferator c-kit [67, 68]. As a result, secondary lymphoid structures, including cryptopatches and innate lymphoid follicles, are absent from the gut of AhR-deficient mice, which show susceptibility to *C. rodentium*. ILC3s feature the secretion of IL-17 and IL-22 [69]. AhR-deficient mice have elevated susceptibility to infection by *C. rodentium*, as well as dextran sulfate sodium (DSS)-associated colitis. DSS can damage the intestinal epithelium and induce inflammatory reactions and microbial dissemination. AhR-deficient mice containing wild-type IELs are resistant to DSS colitis, indicating IEL role in injury reduction.

AhR-deficient mice have lower amounts of skin and intestinal IELs and intestinal ILCs, thereby increasing susceptibility to *C. rodentium* infection. These cell types, and the generation of normal gut lymphoid follicles, are regulated by AhR ligands in the diet. In addition, activation of AhR by microbial products equally regulates the production of DP IELs, which constitute another critical group that controls intestinal immunity [70]. It may also be due to the lack of IL-22 that affects the commensal flora [71]. In fact, ID2, a transcription factor, regulates the expression of IL-22 in ILCs via AhR- and IL-23-dependent mechanisms, thereby modulating the intestinal colonization of *C. rodentium* [72]. In addition, AhR also controls the production of IL-22 by Th22 cells, which protect against intestinal pathogens [73, 74]. All these data suggest AhR has a critical function in controlling the interaction at environmental interfaces with microorganisms by regulating IL-22 and other cellular factors. Interestingly, *cyp1a1* overexpression leads to the exhaustion of physiological AhR ligands and also increases susceptibility to intestinal bacterial infections [75], highlighting that AhR ligand availability and metabolism are important in controlling AhR-dependent immune effects.

8. AhR and T cells

AhR plays an important role in controlling adaptive immunity, and regulating T cell differentiation and direct or indirect functions by affecting antigen presenting cells. It was found that TCDD-activated AhR could inhibit the immune response [76], which is subsequently associated with CD4^{+} T cell induction [77–79]. In addition, the role of AhR in Th17 function and T cell-induced IL-22 biosynthesis have also been determined [74, 80–83].

8.1 AhR and regulatory T cells (Tregs)

AhR shows high expression in Th17 cells, undetectable amounts in Th1 and Th2 cells, and low expression in Tregs. Tregs constitute a T cell subgroup, which helps

maintain tolerance to autoantigens, preventing autoimmune pathologies. FoxP3$^+$ Tregs [84, 85] and IL-10-producing type 1 regulatory T cells (Tr1 cells) [86] are the most typical Treg entities. Foxp3$^+$ Tregs and Tr1 cells are associated with AhR.

TCDD, ITE, KYN and laquinimod derivatives activate AhR, thus increasing FoxP3$^+$ Treg amounts via various mechanisms, e.g., by directly activating epigenetic modifications that regulate Foxp3 transcriptionally and via DC regulation [80, 87–92]. In the presence of TGF - β 1, activating AhR with TCDD can also upregulate SMAD1 in human Tregs, resulting in stable expression of FoxP3 [93]. It was shown in mice with AhR-deficient T cells that AhR could also inhibit the activation of STAT1, which in turn inhibits FoxP3$^+$ Treg differentiation [94]. In addition, AhR regulates the epigenetic modifier Aiolos, which downregulates genes associated with T cell's effector function, such as IL-2 [87]. However, the effect of AhR on FoxP3$^+$ Tregs may be affected by the applied experimental model, which may reflect the different effects of tissue-specific action and/or AhR agonist provided by the symbiotic flora [95].

Tr1 cells participate in controlling tissue inflammation via IL-10 secretion. IL-27 promotes the differentiation of Tr1 cells [96–98], while IL-21 plays an autocrine role in their stabilization [98, 99]. IL-27 upregulates AhR in Tr1 cells via STAT3. Then, AhR amounts are maintained by transactivation of the AhR promoter by AhR itself [100–102]. The important role of AhR in Tr1 cells *in vivo* is reflected by insufficient Tr1 cell differentiation induced by long-term anti-CD3 treatment of AhR-mutant mice.

AhR triggered CD39 equally affects Tr1 cell differentiation. After induction, T cells secrete eATP [103], which then interferes with the differentiation of Tr1 cells through hypoxia inducible factor-1α (HIF1-α). HIF1-α binding is superior to the interaction between AhR and ARNT, and promotes the degradation of AhR through the immune proteasome, thus inhibiting the differentiation of AhR dependent Tr1 cells [101]. The expression of CD39 driven by AhR can deplete eATP and promote the differentiation of Tr1 cells. Therefore, AhR regulates central genes in the Tr1 cell transcription program, while limiting the inhibitory effect of eATP-dependent HIF1-α induction on Tr1 cell differentiation. Overall, the above findings confirm AhR as a potential therapeutic target for immunomodulation.

8.2 AhR and T helper 17 (Th17) cells

Th17 cells, forming a unique CD4$^+$ T cell subgroup, can biosynthesize Th17 cytokines and play key roles in the pathogenetic mechanisms of multiple inflammatory ailments. Their differentiation is triggered by IL-6 and transforming growth factor-beta (TGF-β). AhR can modulate Th17 cells by binding to the DRE site in the IL-17 promoter. In addition, AhR and STAT3 can synergistically upregulate Aiolos (IKZF3), an Ikaros family member, which can decrease the expression of IL-2, thus increasing Th17 cell amounts [64].

Th17 cells, producing IL-17A and expressing ROR-γt, are involved in immune responses to extracellular bacterial and fungal pathogens, and participate in the pathological mechanisms of multiple autoimmune diseases [104, 105]. Their differentiation involves joint effects of TGF-β and IL-6 or IL-21 [106–108]. AhR shows high expression in Th17 cells and is activated by FICZ, which can enhance Th17 cell differentiation and promote IL-22 expression. On the contrary, AhR deficiency can cause Th17 cells to produce IL-22, which may reflect AhR's function in promoting RORγt recruitment to the IL-22 promoter.

8.3 AhR and other T cells

Th22 cells are a CD4$^+$ T cell subpopulation. They produce IL-22 without IL-17's intervention and their differentiation is induced by IL-6, IL-21 or IL-23. AhR

controls the production of IL-22 in Th22 cells, and other cellular factors are essential for their mucosal immune functions [73, 109–111].

The AhR pathway also significantly affects $CD8^+$ T cells. Activation of AhR by TCDD indirectly inhibits the primary response of $CD8^+$ T cells to influenza virus through the regulatory mechanism of DC function [112]. In addition, $CD8^+$ T cells of mouse models administered the AhR agonist TCDD in the developmental stage show a weak response to influenza virus infection later in life [113]. The above data indicate epigenetic alterations that can lead to prolonged functional defects in $CD8^+$ T cells detectable after viral attack. Compared with other $CD8^+$ T cell subsets, AhR expression is much higher in tissue resident $CD8^+$ memory cells (TRMs). Taken together, these findings indicate that, similar to previously reported $CD4^+$ T cell data, the AhR pathway plays a major role in regulating specific $CD8^+$ T cell subgroups, such as TRMs and DP IELs.

AhR equally regulates γδ T cells, which are tissue resident lymphocytes. It regulates first-line immune response at epithelial sites and controls tissue homeostasis [114]. Despite AhR expression in the totality of γδ T-cell subgroups, AhR-deficiency significantly reduces the amounts of skin intraepithelial lymphocytes, mostly composed of Vγ3 and Vγ5 γδ T cells in the intestine and CD8αα αβ T cells [115]. AhR also regulates IL-22 expression by γδ T cells that produce IL-17 [116, 117]. The above data indicate that AhR has a significant effect on T cells residing in tissues, which supports further investigation of AhR's function in non-$CD4^+$ T cells.

In conclusion, AhR controls T cell responses at many levels and regulates transcription factors, enzymes, epigenetic modifiers and effector molecules that modulate T cell stability and metabolism. Lineage-specific responses to AhR induction may lead to ligand-specific effects, which are combined with cytokine-driven activities on the genome, thereby regulating AhR-interacting chaperones and controlling the accessibility of AhR's direct and indirect transcription targets [118]. Comprehensive studies of these interactions should provide insights into the design of immune-modulators against AhR.

9. AhR and B cells

The B lymphocyte is an important part of humoral immunity, which has high specificity against a variety of pathogens. After stimulation via an antigen receptor, activation of immature B cells leads to clonal expansion, antibody isotype conversion and differentiation into antibody-secreting plasma cells, thus producing strong immune reactions [119]. In the process of infection, mature B cells in the lymph nodes and secondary lymphoid organs undergo somatic hypermutation and produce plasma cells featuring elevated antigen affinity and unique effector function [120].

It seems that all B cells produce AhR, but specific subsets, e.g., marginal B cell and B1 B cell subsets, have higher levels than the others. Li and collaborators demonstrated that AhR contributes to the development of B lymphocytes, based on cord blood CD34 and feeder cells, which promote B cell development. Meanwhile, AhR induction inhibits the formation of early B cells and pro-B cells. AhR controls B cell differentiation by transcriptionally suppressing the early B cell genes EBF1 and PAX5 [121].

AhR, overtly induced after activation of B cells, has a critical function in regulating the fate of activated cells. Vaidyanathan and colleagues revealed AhR suppresses switch-like recombination by changing the amounts of activated cytidine deaminase. These authors showed that AhR suppresses B cell transformation into plasmablasts and plasma cells that secrete antibodies [122]. In addition, Villa et al.

provided evidence of a role for AhR in B cells, revealing that AhR expression is increased after administration of IL-4 as well as B cell receptor engagement. Nevertheless, the proliferation of AhR-deficient B cells is decreased, and cells could not progress to the S-phase. Furthermore, AhR-deficient B cells could not compete with the decreased $AhR^{+/+}$ B cell capability of reconstructing the empty host, and could not induce antigen-dependent proliferation in mice. Gene expression profile analysis showed that AhR excision downregulates cyclin O, an important gene controlling the cell cycle [123].

10. AhR and dendritic cells (DCs)

DCs are essential in controlling T cell response and regulating immune tolerance [124]. AhR regulates DC differentiation and function, thereby profoundly affecting T cell-dependent immune reactions. AhR also affects antigen presentation by DCs. Bone marrow derived DCs (BMDCs) exposed to TCDD show decreased CD11c amounts, but increased production of MHC-II, CD86, IL6 and TNFα [125]. Similar findings were reported in TCDD treated splenic DCs [126]. However, different results were observed by using the AhR agonist ITE. The expression of MHC-II and co-stimulatory molecules and the production of Th1 and Th17 polarization cytokines in splenic DCs were decreased by ITE stimulation of AhR.

Recent experiments in ovalbumin-induced asthma models provide additional evidence for the physiological regulation of AhR in DCs, with AhR-deficient mice exhibiting enhanced inflammatory reactions, elevated Th2 differentiation and higher DC MHC-II and CD86 amounts [127]. In addition, AhR signaling has been reported to regulate the activity of $CD103^+/CD11b^+$ DCs during influenza virus infection, thereby reducing induction in protective $CD8^+$ T cells [128]. Overall, this evidence confirms that AhR is a potential therapeutic target for regulating T cell responses in DC.

Multiple mechanisms are involved in AhR-associated regulation of DC function. AhR upregulates IDO 1 and 2 [129, 130], which catalyze the production of KYN, thus promoting the differentiation of $FoxP3^+$ Tregs [131]. Indeed, AhR-deficient DCs could not induce Treg differentiation and Th17 cell proliferation in culture. It is consistent with the immunosuppressive effect of AhR in DCs. Recently, it was reported that IDO expression is maintained by an autocrine loop involving AhR and KYN in tumor infiltrating tolerogenic DCs [132]. Additionally, AhR induction in DCs induces a retinoic acid-dependent enzymatic mechanism, thus promoting $FoxP3^+$ Treg differentiation and inhibiting effector T cells [133–137].

11. Conclusions

Studies evaluating AhR's functions in immune cell development, immune response modulation and immune tolerance have aroused great interest. Originally, AhR was considered a protein sensing environmental substances and regulating drug metabolism. Recently, the role of AhR in regulating normal physiological processes has attracted increasing attention. The organism must perceive and mount substantial responses to environmental changes. Indeed, AhR senses biochemical, chemical and physical environments. Combined with a small amount of high-affinity physiological ligands, including FICZ and ICZ, AhR plays a role in cell proliferation, differentiation and function.

Current evidence indicates that AhR has a critical function in host response to bacterial pathogens. It also overtly influences resistance to infections by

extracellular and intracellular bacteria. AhR is considered the best resistance factor for LM. It may have a new function in the innate immunity of LM infection, and AhR-deficient mice have increased sensitivity to LM. Activation of AhR can protect mice from the deadly attack of *S. pneumoniae*, inhibit bacterial growth and fight infection. AhR can also react with viral pathogens and parasitic infections. After infection by viruses and parasites, lack of AhR aggravates the host's inflammatory response. AhR regulates host's immune cells, confirming that AhR is a regulatory molecule with essential functions in the activation and induction of immune cells, e.g., T cells and inflammatory factors. Barrier organs are critical in immunity; specifically, large amounts of *ahr* are expressed in the intestine, which has a high potential for preventive and treatment interventions. AhR has a critical function in controlling the degree of inflammation in response to symbiotic microbiota and tissue destruction. Progress is being made in determining the molecular mechanisms by which AhR affects different cell types. To understand the complex process of AhR in immunity and antibacterial, to mitigate risks, and to develop novel treatment and prevention tools, more research is needed.

Abbreviations

AhR	aryl hydrocarbon receptor
PAS	pern-arnt-sim
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
ARNT	aryl hydrocarbon receptor nuclear Translocator
AhREs	AhR response elements
DREs	dioxin
XREs	xenobiotic
CYP1A1	cytochrome P450 family 1 subfamily A member 1
AhRR	aryl hydrocarbon receptor repressor
TDO2	2,3-dioxygenase
IDO	2,3-dioxygenase
KYNU	kynureninase
KMO	kynurenine 3-monooxygenase
KYN	kynurenine
DBD	the amino-terminal DNA binding domain
Bhlh	basic helix–loop–helix
NLS	the nuclear localization signal
TAD	the transactivation domain
LM	*Listeria monocytogenes*
IL-6	interleukin 6
TNF-α	tumor necrosis factor alpha
NF-κB	the nuclear factor kappaB
CFUs	colony forming units
MCP-1	monocyte chemotactic protein 1
IFN-γ	interferon γ
ROS	reactive oxygen species
BPI/LBP	bactericidal/permeability-increasing protein / lipopolysaccharide-binding protein
LPS	lipopolysaccharide
NLRP3	NLR family pyrin domain containing 3
3-Mc	3-methylcholine
FICZ	6-formylindolo[3,2-b]carbazole
HSV	herpes simplex virus

iNOS	inducible nitric oxide synthase
DCs	dendritic cells
ILC	lymphoblastoid cell
RegIIIg	Type III Reg gamma
IELs	intraepithelial lymphocytes
ILC3s	group 3 Innate lymphoid cells
DSS	dextran sulfate sodium
Tr1 cells	type 1 regulatory T cells
HIF1-α	hypoxia inducible factor-1α
Th17	T helper 17
TGF-β	transforming growth factor-beta
TRMs	tissue resident $CD8^+$ memory cells
DCs	dendritic cells
BMDCs	bone marrow derived DCs

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Chapter 5

Metabotropic Receptors 4 and the Immune Responses

Zhuoya Wan and Song Li

Abstract

Neurotransmitters (NTs) have recently received increasing appreciation as important immune modulators. The immune cells express receptors for many classes of NTs and the communication between NTs and their receptors establish neuro-immune interactions for regulating effective immune response in both central nervous system (CNS) and peripheral tissues. Metabotropic Glutamate Receptor 4 (mGluR4) is expressed at high level in CNS and plays a role in various physiological and pathophysiological processes in CNS. Recently, mGluR4 has been reported to be expressed on immune cells and have an impact on regulating the immune system. This chapter summarized the works associated with the immunogenic function of mGluR4 and its potential underlying mechanisms.

Keywords: metabotropic glutamate receptor (mGluR4), immune response, peripheral tissues, central nervous system (CNS), cancer, autoimmune diseases

1. Introduction

Neurotransmitters (NTs) have recently received increasing appreciation as important immune modulators. The immune cells express receptors for many classes of NTs and the communication between NTs and their receptors establish neuro-immune interactions for regulating effective immune response in both CNS and peripheral tissues [1]. Interestingly, the role of NTs is very complicated and the same NTs can even exert opposing effects for promoting or inhibiting tissue immunity in different contexts [2–6].

Studies of the NTs and their receptors in modulating immunity are limited and therein are important areas of investigations. L-Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian CNS [7]. It acts via two classes of receptors, ligand gated ion channels (ionotropic receptors (iGluRs))-regulating rapid responses upon activation, and G-protein coupled (metabotropic) receptors-modulating signal transduction cascades. Eight different types of mGluRs, mGluR1 to mGluR8 are divided into groups I, II, and III on the basis of their intracellular signal transduction mechanisms, agonist pharmacology, and sequence homologies (see **Figure 1**) [8]. Group I includes mGluR1 and mGluR5, coupled to Gq protein; group II includes mGluR2 and mGluR3, coupled to Gi and Go proteins; group III includes mGluR4, mGluR6, mGluR7 and mGluR8, coupled to Gi and Go proteins in heterologous expression systems.

mGluR4 is expressed at high levels in CNS and plays a role in various physiological and pathophysiological processes in CNS [9, 10], such as learning, memory, and cognitive impairment. In addition, growing evidence indicates that mGluRs

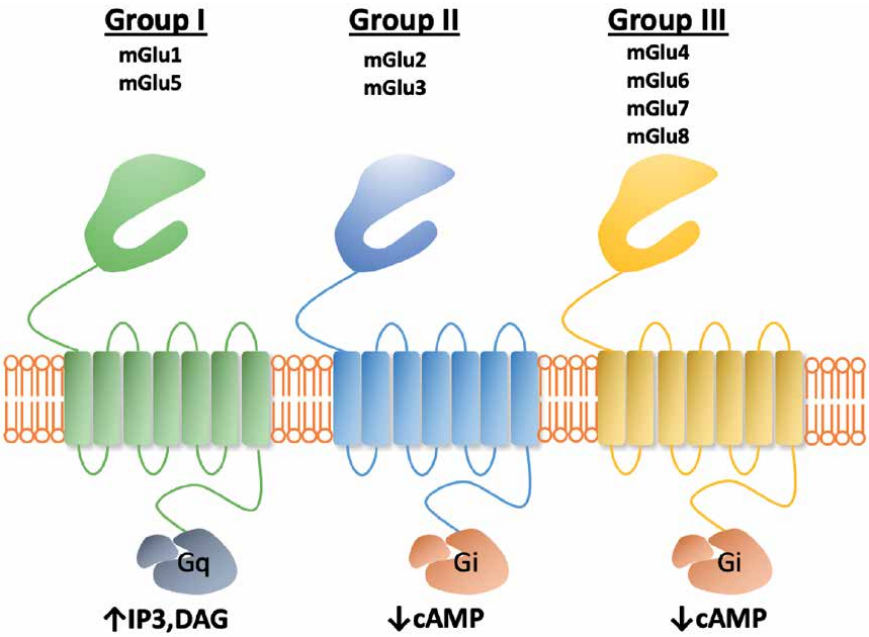

Figure 1.
The summary of mGluRs families. mGluRs are classified into three families: group I, group II, and group III. In the CNS, activation of mGluRs from group I leads to the induction of phosphoinositide hydrolysis with formation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). The activation of groups II and III receptors induce a decrease on the intracellular levels of cyclic adenosine monophosphate (cAMP) [7].

are expressed in the peripheral such as thymus and lymphocytes [11]. These results suggest a potential role of mGluR4 in immune regulation. In this chapter, we summarized the association of mGluR4 with immune responses and its role in different diseases. The potential of mGluR4 as a novel therapeutic target in immune-related diseases was also discussed.

2. Expression of mGluR4 on immune cells

Clinical data indicated that elevated plasma concentrations of Glu are associated with immune deficiency [12, 13]. In addition, in vitro assays showed that high concentration of Glu (>100 uM) can inhibit mitogen-induced T-cell proliferation [12, 13]. Therefore, it is not surprising that immune cells express mGluRs. It has been proposed that mGluRs can mediate an emergency mechanism once high levels of Glu are reached.

Using immunostaining and Western blot analysis, Rezzani et al. observed the expression of mGluR4 in rat thymic cells [14]. The expression of mGluR4 was abundant in dendritic cells (DCs) and lymphocytes of the thymic medulla but was weak in lymphocytes of the cortex. It is interesting to note that a rapid inhibition on the expression of mGluR4 was induced in the rat thymus after treatment with cyclosporine (an immunosuppressant). The mGluR4 expression reached undetectable levels after a longer treatment regimen of cyclosporine.

Other evidence also pointed out that the expression of mGluRs is not exclusive to young immune cells because mature lymphocytes are activated by selective mGluRs ligands. In addition, rat peripheral lymphocytes responded by producing reactive oxygen species (ROS) when they were exposed to the group III mGluRs

agonist L-2-amino-4-phosphonobutyric acid (L-AP4) [15]. ROS play important roles in T-cell biology and participate in activation-induced T cell apoptosis and hence in the termination of the immune response [16]. Moreover, DCs are capable of secreting glutamate when interacting with T lymphocytes, a process that might be essential for the function of lymphocyte. This hypothesis is based on the fact that the absence of glutamate led to impaired Th1 (Interleukin-2 (IL-2) and interferon-γ) and proinflammatory (IL-6 and tumor necrosis factor-alpha) cytokine production. However, these changes were not correlated with a decrease in T-cell proliferation.

3. mGluR4 and Autoimmunity in CNS

A role of mGluR4 in immune modulation was first described in an autoimmune disease model [17]. Fallarino et al. [17] reported that mGlu4 knockout mice ($Grm4^{-/-}$) were highly susceptible to experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis. More specifically, $Grm4^{-/-}$ mice and their wild-type (WT) counterparts were immunized with myelin oligodendrocyte glycoprotein (MOG35–55), which can induce EAE in C57BL/6 mice. The EAE clinical scores were recorded periodically and a lack of mGluR4 was found to be associated with earlier onset, more severe, and ultimately fatal EAE in >40% of the hosts. Along with these changes, white matter demyelination and inflammatory infiltrates were more prevalent in the spinal cord of MOG-vaccinated $Grm4^{-/-}$ mice in comparison to their WT counterparts, according to the morphological changes. The phenotype has also been characterized in littermates as well (heterozygote breeding—with cohorts of mice being matched for gender and age) and the disease indications were also more severe in $Grm4^{-/-}$ and $Grm4^{+/-}$ mice than in WT mice. In contrast, treatment of N-Phenyl-7-(hydroxyimino) cyclopropa [b] chromen-1a-carboxamide (PHCCC), an Grm4-positive allosteric modulator led to increased resistance to EAE. This was in agreement with previous reports demonstrating that long-term treatment of L-AP4 can increase the recovery rate from EAE in Lewis rats [17, 18].

There was significant infiltration of $CD4^+$ T cells, $CD8^+$ T cells and $B220^+$ B cells in both peripheral lymphoid organs and the CNS in both $Grm4^{-/-}$ and WT mice, but the percentages of $CD4^+$ and $CD8^+$ T cells as well as $CD11b^+$ and $CD11c^+$ cells were significantly higher in the CNS of $Grm4^{-/-}$ mice at the peak of disease [17]. Extended studies using littermates from heterozygote breeding further showed that the disease course was more severe in $Grm4^{-/-}$ and $Grm4^{+/-}$ mice than in WT mice. The cytokine profiling of sorted $CD4^+$ T cells from brain-infiltrating leukocytes (BILs) and pooled lymph nodes demonstrated a significant increase in *Rorc* transcripts (encoding the T_H17 specification factor), a reduction in *Foxp3* (Treg) transcripts, and no change in Tbx21 (coding for Tbet; a T_H1 maker) in $Grm4^{-/-}$ mice during the neurologic signs. No changes were observed in *Gata3* (a T_H2 marker) in both groups. These data suggested that $Grm4^{-/-}$ tipped the balance of transcriptional activation in favor of inflammatory genes in response to MOG vaccination. In particular, $Grm4^{-/-}$ favored the emergence of T_H17 over Treg cells, which would sustain inflammation and exacerbate EAE [18].

Expression of mGluR4 was confirmed in several immune subpopulations, such as $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, $B220^+$ B cells, $CD11b^+$ and $CD11c^+$ cells, particularly in $CD4^+$ T and $CD11c^+$ cells, suggesting those cells as potential targets for Grm4 mediated effects. The expression of mGluR4 was also confirmed in DC subsets in splenocytes, including conventional DCs (cDCs; $CD11b^+CD11c^{high}$) and plasmacytoid DCs (pDCs; $mPDCA1^+$ $CD11c^{low}$). They have further shown that

treatment with toll-like receptor ligands such as lipopolysaccharide (LPS) and CpG-oligonucleotide (CpG-ODN) led to increased Grm4 expression. Modulation of mGluR4 expression in activated nTreg cells ($CD4^+$ $CD25^+$) and LPS-stimulated cDCs was also confirmed, further supporting that mGluR4 activation within an immunologic synapse contributes to the crosstalk and reciprocal influence between T and accessory cells [17].

IL-17-producing T helper (Th17) cells are considered mediators of autoimmunity in multiple sclerosis and EAE. The accumulation of Th17 cells in the CNS as well as in the periphery is also associated with the development of demyelinating plaques of multiple sclerosis [19]. Fallarino et al. [17] also pointed out that the absence of mGlu4 in dendritic cells is key to inducing a differentiation of T helper cells toward the Th17 phenotype. More specifically, possible regulatory function of mGluR4 in the interaction between $CD4^+$ T cells and DCs has been examined. Both cDCs and pDCs from $Grm4^{-/-}$ mice produced higher amounts of IL-6 and IL-23, but less IL-12 and IL-27, compared to their WT counterparts in response to LPS or CpG-ODN, respectively [17].

The notable results of coculturing of WT $CD4^+$ T cell and $Grm4^{-/-}$ DCs demonstrated an increase of IL-$17A^+$ $CD4^+$ T cells, along with a significantly reduction of IFN-γ producing $CD4^+$ T cells (a portion of which also expressed IL-17A). However, they failed to see this effect when the coculture consisted of WT DCs and $Grm4^{-/-}$ T cells, suggesting that the effect of mGluR4 depletion was largely dependent on DCs in this *in vitro* system. The cytokine production in culture supernatants has been examined and there are decreased amounts of T_H1-associated IL-2 in coculture system involving $Grm4^{-/-}$ cDCs. IL-27 is known to counter the effect of IL-6 in directing TH17 cell development, which can limit the EAE progression. The decrease in IL-27 during activation of naïve $CD4^+$ T cells might be another reason for favoring the emergence of Th17 cells [17].

They also suggest that activation of mGlu4 (as a result of elevated levels of glutamate during the neuroinflammation) might exert a protective effect by preventing the unbalance in T helper cells. Such mechanism presents a clear therapeutic potential for treating autoimmune related disorders.

The underlying mechanism for Grm4-mediated immune regulation is not clear at present. However, there appears to be a cross-talk and reciprocal influences between Grm4 and indoleamine 2,3-dioxygenase 1 (IDO-1) pathways [20]. IDO1 has been well known to be involved in generating an immunosuppressive environment through catalyzing the metabolism of tryptophan, resulting in tryptophan depletion and accumulation of kynurenine [21]. A protective role of IDO-1 has been shown in mice with different forms of EAE including acute, relapsing–remitting, and adoptively transferred disease [22]. Interestingly, in addition to the direct immunosuppressive effect of kynurenine through inhibition of $CD8^+$ T cells and activation of Treg cells, kynurenine metabolites such as cinnabarinic acid (CA) act as selective, although weak, orthosteric agonists of mGluR4 [23]. The therapeutic effect of CA in acute EAE was attenuated in $Grm4^{-/-}$ mice [24]. On the other hand, activation of Grm4 could positively impact the IDO1 pathway. Treatment of DCs with ADX88178, a positive allosteric modulator (PAM) of Grm4, led to both increased expression levels of IDO-1 and phosphorylation of IDO-1 [20]. These effects require a Gi-independent, alternative signaling pathway that involves phosphatidylinositol-3-kinase (PI3K), Src kinase, and the signaling activity of IDO1. Moreover, the effect of ADX88178 on the expression of several cytokines was impaired in $IDO1^{-/-}$ DCs [20]. Therefore, Grm4 and IDO1 constitute a loop that provides a positive feedback mechanism to amplify the immune-protective effect in EAE and possibly other immune-related diseases [20].

4. mGluR4 and cancers

Most studies on the role of glutamate receptor in cancers have been focused on iGluRs [25, 26]. Tumor cells originated from neuronal tissues express iGluRs subunits and iGluRs antagonists have shown inhibitory effect on the proliferation of the tumor cells. Similarly, iGluRs subunits have been shown to be expressed in several peripheral cancers and blockade of the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) inotropic glutamate receptor subtypes leads to decreased cell proliferation and migration [26].

mGluRs are also expressed in several cell lines derived from human tumors, including neuroblastoma, thyroid carcinoma, rhabdomyosarcoma/medulloblastoma, lung carcinoma, multiple myeloma, glioma, colon adenocarcinoma, astrocytoma, T cell leukemia, and breast carcinoma [27]. In particular, mGluR1 has been shown to be expressed in subsets of human melanomas [28]. Ectopic expression of mGluR1 in melanocytes drives the development of melanoma in mouse models. Pharmacological inhibition of mGluR1 led to inhibition of tumor cell growth both in vitro and in vivo [28]. Riluzole, an antagonist of mGluR1 signaling has advanced to phase II clinical trial in patients with advanced melanoma [29, 30].

The studies on the roles of mGluR4 in cancers are very limited and controversial. Change et al. studied the expression pattern of mGluR4 in several healthy and diseases-derived human tissues [31]. mGluR4 receptor expression was identified in 68% of colorectal carcinomas, 50% of laryngeal carcinoma, and 46% breast carcinomas. In the case of colorectal carcinoma, overexpression of mGluR4 was correlated with poor prognosis, and cell lines derived from human colorectal carcinomas showed increased cell invasiveness when treated with L-AP4. In another study, comparative proteomics was used to characterize a human colon cancer cell line that was resistant to 5-fluororacil (5-FU, a common chemotherapy agent). Interestingly, 5-FU resistant cells were found to overexpress mGluR4 in comparison with parental cancer cells. It has been demonstrated that cell survival was increased by the group III mGlu receptor agonist L-AP4 in the nonresistant parent cancer cells; conversely, survival was synergically decreased by 5-FU and the group III receptor antagonist MAP4 in 5-FU-resistant cells. It is noteworthy to mention that 5-FU downregulated mGluR4 expression, and MAP4 has a dose dependent cytotoxic effect in both cell lines [32].

In contrast to the above reports, mGluR4 agonists are shown to inhibit the proliferation of human breast and bladder cancer cells in a GRM4-depenedt manner [33]. In the study by Lasek et al., the expression of mGlu4 was shown to be inversely correlated with the severity of human medulloblastoma [34]. After scoring the extent of immunoreactivity for mGlu4 in human biopsies of medulloblastoma, the absence of spinal metastases, cerebrospinal fluid spread, and tumor recurrence as well as the survival of patients were all shown to be associated with high levels of mGlu4 immunoreactivity. Treatment with PHCCC (which is considered a group I mGlu receptor antagonist but can also act as a positive allosteric modulator of mGlu4 receptor) reduced the proliferation of cultured medulloblastoma cells and inhibited the growth of medulloblastoma implants in mice. In addition, subcutaneous or intracranial injections of PHCCC during the first week of life reduced the incidence of medulloblastoma from 85 to 28% in a mutant mouse model known to develop the disease upon X-ray irradiation. This indicates that activation of mGlu4 receptors also affects early events in tumorigenesis [35].

The above studies focus on the role of tumor cell-derived Grm4. It has been reported that the plasma levels of Glu are generally elevated in patients with carcinoma and seem to correlate with an impairment in immune function [36]. However,

the role of immune cell-derived mGluR including mGluR4 has hardly been studied. Kansara et al. reported that $Grm4^{-/-}$ mice showed accelerated radiation-induced tumor development in an irradiation-induced osteosarcoma model [37]. Outside the CNS, mGluR4 is highly expressed by DCs, as well as $CD4^+$ T cells [17]. In the mouse osteosarcomas, they found that mGluR4 is predominantly expressed by $CD45^+CD11c^+MHC^+$ myeloid cells within the tumor microenvironment (TME) instead of tumor cells. Few $CD4^+$ T cells were detectable to characterize mGluR4 expression. In consistent with the study by Fallarino et al. [17] in an EAE model, $Grm4^{-/-}$ DCs isolated from the tumors showed increased expression of IL-23. Interestingly, high expression of IL-23 has been observed in primary osteosarcomas and allografted cell lines relative to normal bone, while ex-vivo cultured osteosarcoma cell lines and primary tumor cells did not express IL23. A role of IL-23 in tumorigenesis has been well established from previous studies [38]. Indeed, $IL23^{-/-}$ mice were resistant to the irradiation-induced osteosarcoma. They hypothesized that knockout of Grm4 in DCs facilitates the oncogenesis of osteosarcoma through increased production of IL-23 [37].

We have recently shown in three murine syngeneic tumor models (B16, MC38, and 3LL) that either genetic knockout ($Grm4^{-/-}$) or pharmacological inhibition led to significant delay in tumor growth (Wan et al., unpublished data). Mechanistically, perturbation of GRM4 resulted in a strong anti-tumor immunity by promoting nature killer (NK), $CD4^+$ and $CD8^+$ T cells toward an activated, proliferative, and functional phenotype. We have further shown that the antitumor activity of Grm4 antagonists can be further improved through combination with anti-PD-1 antibody. The differing role of Grm4 in different tumor models may reflect the complex functions of Grm4 in different tumor environment. More studies are needed to further define the roles of immune cells-derived Grm4 and its potential as a novel therapeutic target for cancer immunotherapy.

5. Conclusions

Although the neurological function of GRM4 in CNS has been well established, its role in modulating immune response just began to be appreciated. GRM4 is expressed in various immune cells and loss of GRM4 function in immune cells led to sensitization to EAE. GRM4 selective agonists may hold potential as a novel therapy for autoimmune disorders of CNS. GRM4 is also expressed in various cancer cells, however, conflicting results have been reported regarding whether GRM4 promotes or inhibits tumor cell proliferation. The role of immune cells-derived GRM4 in antitumor immunity is also controversial and may reflect the complex function of GRM4 in different tumor microenvironment. Further studies using more defined animal models and selective GRM4 modulators may not only advance our understanding of the complex immunobiology of GRM4 but also lead to the development of a new immunotherapy for the treatment of cancer.

Acknowledgements

This work was supported, in part, by NIH grant RO1 CA219399.

Conflict of interest

The authors declare no conflict of interest.

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Edited by Maria del Mar Ortega-Villaizan and Veronica Chico

Infectious microbial agents such as viruses, bacteria, fungi, and parasites can cause pathological disorders and even death in organisms exposed to the environment. However, organisms have an immune system to control infection caused by pathogens. The immune system is divided into the innate and the adaptive immune systems. The innate immune system is the first mechanism to respond to infections, whereas the adaptive immune system is based on immune memory. This book provides an overview of antiviral and antibacterial immune responses in different immune-reactive organs and across different animal species, from higher to lower vertebrates.

Published in London, UK

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ISBN 978-1-83968-784-6
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