Doctoral thesis from the Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden

Immunohepatotoxicity of the persistent environmental pollutants perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS)

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Stockholm 2011
TO MY PARENTS

TO MY FAMILY
List of Publications

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:


II. Qazi MR, Nelson BD, DePierre JW, Abedi-Valugerdi M. 28-Day dietary exposure of mice to a low total dose (7mg/kg) of perfluorooctanesulfonate (PFOS) alters neither the cellular compositions of the thymus and spleen nor humoral immune responses: Does the route of administration play a pivotal role in PFOS-induced immunotoxicity? *Toxicology.* 2010; 267(1-3):132-9.


*These authors have contributed equally to this paper.

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Additional publication and manuscript:


Abstract

Perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS), manufactured for a variety of industrial and consumer applications, are ubiquitous environmental pollutants. Their accumulation in humans and wildlife raises serious health concerns.

Here, we examined the potential effects of PFOA and PFOS on the innate immune system in mice. Short-term dietary exposure to high doses reduces the total number and subpopulations of circulating white blood cells. Moreover, production of proinflammatory cytokines by macrophages in the peritoneal cavity and bone marrow, but not in the spleen following exposure to in vitro or in vivo stimulation by bacterial lipopolysaccharides is enhanced. With respect to adaptive immunity, PFOS reduces the total numbers of thymocytes and splenocytes and subpopulations thereof in a dose dependent fashion. Furthermore, comparison of wild-type mice and the corresponding knock-out strain lacking peroxisome proliferator-activated receptor-alpha revealed that these immunological changes are partially dependent on this receptor. Our further studies also show that sub-chronic dietary exposure to an environmentally relevant dose of PFOS does not alter the cellularity of the thymus and spleen and exerts no influence on humoral immune responses.

To facilitate examination of the effects of PFOA and PFOS on the hepatic immune system, we developed a procedure for mechanical disruption that yields a larger number of functionally competent immune cells from this organ. In our last study, lower doses of PFOA or PFOS induced hypertrophy of hepatocytes and altered the hepatic immune status. Thus, we find that short-term, high- and low-dose exposure of mice to these fluorochemicals is immunohepatotoxic.
**Sammanfattning**

Perfluorooctanat (PFOA) och perfluorooctansulfonat (PFOS) som tillverkas för många olika industri och konsumentprodukter, är globalt förekommande miljögifter. Deras ackumulering i människor och djur ger upphov till en stark oro för hälsoproblem.

Vi har granskat effekterna av PFOA och PFOS på det medfödda, ospecifika immunförsvaret. Exponering för höga doser via maten under kort tid minskar det totala antalet cirkulerande vita blodkroppar samt delpopulationerna. Immunsvar ökar dock efter stimulering med bakteriella lipopolysaccharider både \textit{in vitro} och \textit{in vivo}, dvs produktionen av proinflammatoriska cytokiner av makrofager i bukhålan och bennärjen, men inte i mjälten ökar. När det gäller adaptiv, specifik immunitet minskar PFOS det totala antalet tymocyter och splenocyter och deras olika subpopulationer. Vid exponering för lägre doser av PFOS induceras hepatomegali utan att påverka tymus eller mjälten. Vi kunde visa att peroxisomal proliferator-aktiverad receptor-alfa medierar effekterna utav PFOS i tymus samt delar av effekterna av PFOS i mjälten genom att använda möss som saknade denna receptor. Detta stöds av vår studie med subkronisk exponering för en miljömässig dos av PFOS vilken inte ändrade den cellulära sammansättningen i vare sig tymus eller mjälte och inte hade något inflytande på det humorala immunsvaret.

För att underlätta studier av hur PFOA och PFOS påverkar immunsystemet i levern utvecklade vi en metod för framrening av immunceller via mekanisk sönderdelning av levern, vilket gav ett större antal av funktionella immunceller från detta organ. I vår sista studie kunde vi påvisa att lägre doser av PFOA eller PFOS inducerade hypertrofi av hepatocyter samt en påverkan av leverns immunförsvar.
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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell-receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
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<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<tr>
<td>IFN-(\gamma)</td>
<td>Interferon-gamma</td>
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<tr>
<td>IHIC</td>
<td>Intrahepatic immune cell</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MZ</td>
<td>Marginal zone</td>
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<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>PFAA</td>
<td>Perfluoroalkyl acid</td>
</tr>
<tr>
<td>PFOA</td>
<td>Perfluorooctanoate</td>
</tr>
<tr>
<td>PFOS</td>
<td>Perfluorooctane sulfonate</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cell</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>TD</td>
<td>T cell-dependent</td>
</tr>
<tr>
<td>TI</td>
<td>T cell-independent</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNP</td>
<td>Trinitrophenol</td>
</tr>
<tr>
<td>Th cell</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>Tumor necrotic factor-alpha</td>
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1. Introduction

1.1 General background

Immunotoxicology can be defined as the study of adverse effects on the immune system resulting from occupational or environmental chemicals, inadvertent or therapeutic exposure to drugs, or, in some instances, biological materials. Studies on both humans and other animals reveal clearly that the immune system is sensitive to a variety of factors, and that damage to this system can be associated with morbidity and even mortality. Regulatory agencies have recognized this fact and testing for immunotoxicity has become a routine component of safety evaluations for therapeutic agents, biological agents, and chemicals.

2. The immune system

Historically, the collective and coordinated response of the cells and molecules that constitute the immune system has been viewed as providing protection from disease and, more specifically, from infectious disease [1]. We now know that many of the mechanisms responsible for resistance to infections can respond to non-infectious foreign antigens and, sometimes, even to endogenous epitopes. There are two fundamentally different types of responses to invading microbes, i.e., the innate and adaptive immune responses. Innate responses are activated, within 4 hours after infection or tissue injury, in attempt to immediately inhibit and control the invading pathogen [2]. The adaptive immune response takes three to five days to produce sufficient numbers of effector cells directed towards specific targets and to develop an immunological memory which recognizes and helps prevent later infection with the same microorganism [3].

2.1 The lymphoid organs

The morphologically and functionally diverse organs and tissues that contribute to the development of immune responses are divided into primary and secondary lymphoid organs. The thymus and bone marrow, where maturation of lymphocytes takes place, are the primary (or central) lymphoid organs. The secondary (or peripheral) lymphoid organs, where lymphocytes interact with antigens, include lymph nodes, the spleen, and various
mucosal lymphoid tissues, including bronchus-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT) [4].

**The bone marrow**

The complex bone marrow (BM) acts both as the site of hematopoiesis and a fat depot. After birth and throughout life, cells of the innate and adaptive immune systems are generated from the hematopoietic stem cells (HSCs) in the mammalian BM (Figure 1) [5], originating primarily from two separate progenitors, i.e., common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) [6]. Under the influence of the microenvironment provided by stromal cells, CMP can further develop into either megakaryocyte-erythrocyte progenitors (MEP) or monocyte-granulocyte progenitors (MGP).

![Figure 1. Hematopoietic cell differentiation in the bone marrow (adapted from [5]).](image-url)
These MEPs can subsequently progress to become erythrocytes or megakaryocytes, while further differentiation of MGP eventually produces granulocytes (neutrophils, eosinophils and basophils) and monocytes.

On the other hand, in the presence of stromal cells and the cytokines they produce, CLP can give rise to mature B and natural killer (NK) cells. This development to mature B cells proceeds through several stages (i.e., pro-B, pre-B, immature B and mature B cells), each of which is characterized by the expression of distinct cell surface molecules, including the B cell antigen receptor, IgM [6-8]. Although the precursors of T cells also originate from HSCs in the BM, development of these cells is completed in the thymus [9].

The thymus

The thymus, the site of T cell development and maturation, is a flat, bilobed organ situated above the heart. Each lobe is organized into two compartments: the outer cortex is densely packed with immature T cells, called lymphocytes; while the inner medulla is sparsely populated with thymocytes [10].

T cell differentiation and selection in the thymus is a multi-step process: the entry of lymphoid progenitor cells; generation of CD4⁺CD8⁺ (cell surface marker express on immature T cells) thymocytes in the outer cortex; positive selection of these double-positive thymocytes; negative selection of CD4⁺CD8⁺ cells and interaction between positively selected thymocytes and medullary thymic epithelial cells, to complete the development of the thymocytes and ensure central tolerance; and, finally export of the mature either CD4⁺ or CD8⁺ T cells from the thymus [11].

This multi-step process entails strict selection, during which only 1-3% of all the original thymocytes survive. The initial development of the lymphoid progenitors into mature T cells occurs via a pathway involving expression of CD25 and CD44 and proceeds until a developmental checkpoint is reached. At this point only those cells that have succeeded in rearranging the gene encoding the β-chain of the T-cell receptor (TCR) are selected for further differentiation [12].
The spleen

The spleen is the largest peripheral lymphoid organ in both primates and rodents, but, in contrast to other such organs, lacks afferent lymphatics and is supplied by the splenic arteries. The spleen is divided into the red pulp (which surrounds the sinusoids and is full of erythrocytes), that filters old or damaged erythrocytes out of the blood, and the white pulp (containing lymphoid follicles, the marginal zone and the periarteriolar lymphoid sheath), which is composed of innate and adaptive immune cells. The cells in the white pulp are organized in a highly specialized manner, optimized for efficient antigen-specific activation of T and B cells [13]. Follicular B cells circulate between the blood and secondary lymphoid organs; express IgD at high levels, and participate in classic adaptive T cell-dependent immune responses. The B cells in the marginal zone are referred to as innate-like and participate preferentially in T cell-independent immune responses.

The structure of the spleen enables it not only to remove senescent erythrocytes from circulation, but also leads to the efficient removal of blood-borne microorganisms and cellular debris. This function, in combination with its highly organized lymphoid compartment, makes the spleen the most important site for immune reactivity towards different types of microbes including fungi [13,14].

2.2 The innate immune system

Most of our encounters with microorganisms do not result in disease. The few microbes that manage to cross the barrier formed by the skin, mucus, cilia and gastric pH are usually eliminated by innate immune mechanisms, which commence immediately upon entry of the pathogen [15]. If phagocytosis cannot rapidly eliminate the pathogen, inflammation is promoted by the production of cytokines and acute phase proteins. This early response is not antigen-specific nor does it generate immune memory [15]. Only when the inflammatory process is unsuccessful in eliminating the pathogen, the adaptive immune system is activated to produce armed effector cells, a process which requires several days. The various innate cell populations, such as neutrophils, monocytes, macrophages, dendritic cells (DCs) and natural killer cells, involved in the phagocytic
uptake of pathogens and endogenous waste, triggered by, e.g., pattern-recognition, complement and Fc receptors, are described below [16].

**Neutrophils**

Neutrophils, together with eosinophils and basophils, belong to the group of leukocytes referred to as granulocytes, since they contain intracellular granules. Neutrophils are also called polymorphonuclear (PMN) cells due to the segmentation of their nucleus into several lobes. These, the most abundant white blood cells, are the first to accumulate at sites of inflammation.

After approximately five days of proliferation and final maturation in the bone marrow, mature neutrophils are released into the peripheral circulation [17]. Once activated in response to acute infection, they migrate to the site of the infection, where they phagocytize foreign antigens and exert anti-microbial action through the production of reactive oxygen species (ROS) and release of toxic components of their granules including α-defencin, lactoferrin, cathepsin and myeloperoxidase [18]. Following these inflammatory responses, neutrophils undergo apoptosis and their debris is processed by macrophages. Neutrophil trafficking from the bone marrow is regulated by the granulocyte colony-stimulating factor (G-CSF), which also attenuates neutrophil apoptosis at local inflammatory sites [19].

**Macrophages**

Macrophages are a ubiquitously distributed population of mononuclear phagocytes involved in numerous homeostatic, immunological and inflammatory processes. Their wide tissue distribution allows them to provide an innate defense against foreign elements prior to the immigration of lymphocytes. Following stimulation, macrophages release the soluble factors interleukin (IL)-1β and tumor necrotic factor-alpha (TNF-α), which activate the innate immune system. Macrophages also release IL-6 and all of these proinflammatory cytokines induce an acute phase response, which is crucial to subsequent activation of adaptive immune responses. Interestingly, activation of the adaptive immune cells feedback regulates macrophages, as exemplified by the
production of interferon-gamma (IFN-γ), originally called macrophage-activating factor, by activated T cells [20].

Macrophages differentiate from monocytes, which migrate from the peripheral circulation into tissues, both under physiological conditions and in response to inflammation [21]. Monocytes develop, as mentioned above, from HSCs in the BM. During monocyte development, myeloid progenitor cells (referred to as granulocytes/macrophage colony-forming units) give rise sequentially to monoblasts, pro-monocytes and, finally, monocytes, which are then released from the bone marrow into the bloodstream [21]. In response to proinflammatory, metabolic and immune stimuli, monocytes migrate from the blood, into the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum in order to replenish long-lived tissue-specific populations of macrophages [22].

The heterogenous morphology of macrophage populations (Figure 2) [23] is reflected in their functions and biochemical properties. Their developmental requirements have been employed to distinguish between classically activated (M1) and alternatively activated (M2) macrophages. The former are activated through toll-like receptors (TLR) that bind components of bacterial cell walls and interferon-γ (IFN-γ), an inflammatory cytokine); whereas the latter are activated by IL-4 or IL-13. A slightly different
classification, based on involvement in host defenses, wound healing and immune regulation, has also been proposed [21].

**Natural killer (NK) cells**

NK cells are cytotoxic lymphocytes that play an important role in innate immunity by lysing tumor or pathogen-infected cells. NK cells can also release cytokines, including IFN-γ, TNF-α, granulocyte-macrophage colony stimulating factors (GM-CSF) and chemokines. These cells are activated by cytokines, following which they bind to the Fc portion of antibodies and exert antibody-mediated cellular cytotoxicity that disrupts infected cells. Regulation of this activity is achieved through so called "activating and inhibitory receptors" that help to differentiate between infected and non-infected cells [24].

The two major subsets of NK cells in humans are distinguished on the basis of the level at which they express CD56 (CD56\textsuperscript{bright} and CD56\textsuperscript{dim}, respectively). CD56\textsuperscript{dim} NK cells are more numerous and excel in cytotoxicity, whereas the CD56\textsuperscript{bright} cells are less cytotoxic, but more potent producers of cytokines [25,26].

**Dendritic cells (DCs)**

DCs were first described by Ralph Steinman nearly 30 years ago [27]. They are professional antigen-presenting cells, specialized in capturing, processing and presenting antigens to immune cells, such as, T cells [28]. They are distributed throughout the body, particularly at sites of pathogen encounter, such as skin and mucosal surfaces. Upon antigen encounter, DCs become activated and thereafter travel to secondary lymphoid tissues, such as the spleen and lymph nodes, where they stimulate naïve T-cells to differentiate and proliferate into functionally competent effector T cells.

DCs play an important role in activating and differentiating B cells by interacting with CD40-activated naïve B cells or by producing cytokines such as IL-12. In the thymus, these cells also have a crucial function in induction of immunological tolerance by eliminating T cells reactive towards self-antigens through a process known as negative selection [29,30]. Both mice and human have two major types of DCs; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [31]. Depending on the type of stimuli and which
of these subsets they belong to, these cells express various cytokines, such as IFN-\(\alpha\), IL-10 and IL-12, involved in the induction of immune responses.

2.3 The adaptive immune system

Adaptive immunity involves the development of highly defined immune responses and the formation of memory that enables the body to mount more specific and stronger immune responses upon reencounter with a pathogen [2]. Two major cell types are involved in maintaining adaptive immunity: T cells and B cells. These cells recognize specific structures on pathogens via their different surface receptors and are responsible for the enormous diversity of the adaptive immune system. B cells recognize antigens through B cell receptors (BCR) and are primarily responsible for humoral immunity. The T cells perform cell-mediated immunity through the T-cell receptors (TCR) with unique specificities dependent on interactions with major histocompatibility complex (MHC) molecules [32]. The differentiation of the cells of the adaptive immune system is dependent on cross-talk with cells of the innate immune system, with cytokines acting as key mediators in this context [33]. T- and B-cells share, as mentioned before, common progenitor cells developed from HSCs in the BM [34].

2.3.1 Cell-mediated immunity

Effector T cells, generated in response to antigen, are responsible for cell-mediated immunity. Both activated helper T cells (Th cells) and cytotoxic T cells (CTL) serve as effector cells in cell-mediated immune reactions. Cytokines secreted by Th cells can activate various phagocytic cells, enabling them to phagocytose and kill microorganisms more effectively. Cell-mediated immune responses are important in defending against intracellular bacteria, viruses, and cancer and are responsible for graft rejection [1].

Helper T cells

Helper T cells (Th cells) or CD4\(^+\) T cells (since they express CD4 on their surface) play a significant role in directing and regulating adaptive immune responses.
Activation of Th cells is initiated by and totally dependent on recognition of peptides bound to MHC class II molecules on the surface of the immune cells known as antigen presenting cells (APCs), which can be either macrophages, DCs or antigen-specific B cells. Depending on the effectiveness of immune response and the cytokine environment, CD4+ T cells can be activated to proliferate and differentiate into Th1, Th2 and Th17 cells.

Th1 cells, which secret IL-2 and IFN-γ, are associated with cell-mediated immunity and the latter cytokine also stimulates macrophages to eradicate intracellular pathogens. These same cells also promote inflammation and enhance phagocytosis of antigens opsonized by antibodies [35]. Th2 cells typically produce IL-4, IL-5, IL-10 and IL-13, which participate in activation and differentiation of antibody-producing B cells and play a central role in IgE-mediated allergic disease. These cells also potentiate elimination of parasitic infection [36]. Th17 cells release IL-17 and IL-22 and are involved in immune responses that are essential for protection against extracellular bacteria and fungi as well as in several autoimmune diseases [37].

Upon sensing an antigen, CD4+ T cells produce the key cytokine IFN-γ, which stimulates macrophages to produce antimicrobial components, i.e., ROS and reactive nitrogen intermediates designed to kill the bacteria [33].

**Cytotoxic T cells**

Cytotoxic T cells or CD8+ T cells are MHC class I restricted. They are primary immune effector cells involved in combating viral infection, as well as, possibly, in the elimination of tumor cells and rejection of allografts. Following activation by an MHC class I:peptide complex on APCs, CTL migrate to the periphery to kill infected cells, a process crucial to limiting the growth and spread of infectious microbes. The infected cells display peptide fragments that originate from cytosolic pathogens, in particular viruses [38]. CD8+ T cells express cytotoxic activity by releasing the contents of their granules, including perforin and granzyme, and by triggering Fas-mediated apoptosis [39].

The helper and cytotoxic memory T-cell populations are derived both from naive T cells after these have encountered antigens and from effector cells following antigenic
activation and differentiation. They are long-lived, quiescent cells that respond with heightened reactivity to a subsequent challenge with the same antigen, generating a secondary response [40].

**γδT cells**

Although a minor population in the peripheral blood, T cells that recognize antigen through a γδ cell receptor on their surface constitute a major population among intestinal intraperitoneal lymphocytes. Unlike αβ T cells, which recognize processed antigens associated with MHC molecules, these γδT cells recognize both natural or synthetic non-peptide antigens [41,42], such as heat-shock proteins (in mice) or phosphorylated bacterial metabolites (in humans) respectively, as well as controlling the integrity of epithelia. γδT cells form an entire lymphocyte system that develops under the influence of other leukocytes, in the thymus and in the periphery. They demonstrate the same potent cytotoxic activity as conventional αβ T lymphocytes and, moreover, produce a variety of cytokines, such as keratinocyte growth factor. Little is known about γδT cell activation, but their main role in host immune responses is to coordinate the innate and adaptive immune responses during the early stage of infection [43].

**NKT cells**

NKT cells are innate-type lymphocytes that express both NK and T cell markers such as CD161 (NK 1.1), CD3 and a TCR. These cells are also different from conventional T cells in that they recognize lipid antigens presented by CD1d (an MHC I-like molecule expressed by antigen-presenting cells) rather than MHC-restricted peptides. Antigen-mediated activation of NKT cells induces rapid production of cytokines (within hours), which is indicative of their potent immunomodulatory function.

The size of the NKT cell population varies considerably among individuals, from undetectable (< 0.001 %) to 3 % of all the lymphocytes in the peripheral blood [44]. NKT cells develop in the thymus and, when the TCR that recognizes CD1d is expressed, diverge from conventional T cells at the double positive (CD4⁺CD8⁺) stage [44].
After migrating from the thymus, NKT cells continue to develop in the periphery and eventually populate the blood, liver, lymph nodes and spleen. Cells that express CD1d and are thus able to present antigen to these NKT cells include macrophages, DCs and B cells [45]. Experimentally, NKT cells are most often activated with alpha-galactoceramide (α-GalCer) or by anti-CD3 antibodies. TCR-mediated activation of NKT cells results in rapid production of large amount of cytokines (e.g. IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α etc), induction of cytotoxic activity and up-regulation of costimulatory molecules [45].

2.3.2 Humoral immunity

The humoral immune response (so-called because the antibodies involved are found in the `humours`, or plasma, lymph and tissue fluids) is mediated by antibodies secreted by the cells of the B lymphocyte lineage or B cells. Upon co-stimulation, e.g., by another antigen-presenting cell, such as DCs, B cells are transformed into the plasma cells that secrete antibodies. Th2 cells assist the entire process. Secreted antibodies bind to antigens on the surfaces of invading microbes, such as viruses or bacteria, and promote phagocytosis.

B cells

The ectopic germinal centers (i.e., the non-lymphoid organs: the rheumatoid synovial membrane, thyroid gland, choroid, and lungs) and the lymphoid follicles of secondary lymphoid organs (such as regional lymph nodes, tonsils, spleen, and mucosal lymphoid tissues) are classical sites of B cell activation, differentiation and proliferation [46]. Immature B cells transported via the circulation to the secondary lymphoid organs. Here, when a specific antigen binds to their BCR, these cells become activated. Usually, signaling via the B cell co-receptor (CD19, CD21, CD81), T cells participate via CD40-CD40L interactions and cytokines are also required for this activation [47]. B cells can function as APCs. B cell activation occurs via two different pathways, T cell-dependent (TD) or T cell-independent (TI), depending on the properties of the antigen.
**T cell-dependent B cell activation**

The TD pathway, which is generally associated with memory formation, includes interaction between activated CD4⁺ Th cells and B cells [48]. This interaction takes place in the outer T cell zone in the secondary lymphoid organs and induces further proliferation and differentiation of the B cells. These important interactions involve binding of the antigen, processed and presented in a MHC class II complex by the B cell, to the TCR. In addition, interactions of co-stimulatory molecules contribute to the subsequent activation of both B and T cells. These co-stimulatory molecules and their ligands include CD40:CD40L (CD154), CD28:CD80/CD86 and CD70:CD27 [49,50]. CD40 signaling in B cells is essential for induction of Ig isotype switching and is associated with the formation of memory.

**T cell-independent B cell activation**

TI pathways can be activated by polymeric antigens with repeating structures that induce strong signals due to cross-linking and clustering of BCR molecules [2]. This pathway can be induced either by TI type-1 (TI-1) or TI type-2 (TI-2) antigens. TI-1 antigens, such as lipopolysaccharide (LPS) or flagellin at high concentrations, induce polyclonal B cell activation. TI-2 antigens consist of molecules with highly repetitive structures, such as carbohydrate polymers present on bacterial cell membranes. TI-2 antigens are recognized by specific BCRs, expressed especially by B cell subsets in the marginal zones (MZ) [51].

**Immunoglobulins**

Immunoglobulins (Ig) are antigen–binding proteins both present on the B cell membrane and secreted by plasma cells. When bound to the cell surface, the Ig functions as a receptor involved in differentiation, activation and apoptosis; while the secreted form neutralizes foreign antigens and recruits other effector molecules [52]. The Ig molecule consists of two identical light (L) and two identical heavy (H) chains complexed in a Y-shape. The assembled molecule contains an antigen-binding site (Fab) along with a crystallisable fragment (Fc) that interacts with receptors present on neutrophils,
monocytes, macrophages and complements. The nature of the heavy chain defines the isotypes of Ig i.e., IgA, IgD, IgG, IgE and IgM.

**IgG** is the most abundant isotype, constituting approximately 85% of the total serum Ig in humans, probably due to its long half-life [53,54]. This is the predominant isotype involved in secondary immune responses characterized by high-affinity isotype-switching antibodies [55]. IgG consists of four subclasses, (IgG1, IgG2, IgG3 and IgG4), numbered in the order of decreasing serum concentration [56].

Since **IgM** is the first isotype expressed during B cell development, as well as the major isotype involved in primary antibody responses, the level of this isotype is frequently used as a marker of infection [56]. IgM constitutes approximately 10% of human plasma Igs and is the second most common isotype in the mucosa (after IgA). IgM is particularly effective in neutralizing and agglutinating blood pathogens, especially those residing within cells, thus preventing cell-to-cell transfer [57].

Although the two forms of **IgA** (IgA1 and 2) account for only about 7-15% of the antibodies in human serum, this is the predominant class of antibody in extravascular secretions [56].

Of all the classes of serum Ig, **IgE** has the shortest half-life and is present at lowest concentration, accounting for 0.02% of the total serum antibodies. This isotype is associated with hypersensitivity and allergic reactions, as well as with responses to infection by parasitic worms [56].

### 2.4 Cytokines

Numerous interactions between the cells of the immune system are mediated by soluble proteins called cytokines, all of which are produced in small amounts in response to external stimuli, such as microbes; generally demonstrate a short half-life, and bind to high-affinity receptors on their target cells. While many cytokines act either on the cells that produce them (called autocrine action) and/or on adjacent cells (paracrine action), most enter the circulation and act on distant tissues (endocrine hormonal action) [58].

Cytokines are involved in the regulation of both innate and adaptive immunity. They play an important role in activating macrophages to produce large amounts of other cytokines, as well as in activating and regulating lymphocytes. Depending on the immune
response evoked, cytokines are referred to as Th1- or Th2-associated [59]. The major cytokines involved in immune regulation are discussed below.

**TNF-α**

TNF-α, synthesized primarily by activated macrophages, is a potent mediator of inflammatory and immune functions. Circulating levels of TNF-α are usually very low under healthy conditions, but rise rapidly during an infection or inflammation [60]. TNF-α influences the expression of adhesion molecules, as well as chemokines that attract immune cells to the site of infection. TNF-α functions by forming a trimer with either the TNF receptor 1 (TNFR1) or TNFR2 present on the surface of almost all nucleated cells.

**IL-6**

IL-6 is a multifunctional cytokine whose inflammatory role depends on whether there is an ongoing acute immune response or not. This signal is involved in the development of cells and tissues, as well as a variety of pathological conditions [61]. IL-6 is not produced constitutively, but synthesized by many different types of cell, including monocytes/macrophages, endothelial cells, fibroblasts, adipocytes and myocytes, in response to inflammatory stimuli such as lipopolysaccharide (LPS), TNF and IL-1β [62].

**IFN-γ**

IFN-γ was first identified in the 1960s, on the basis of its distinctive activity against Sinbis virus [63]. A number of both innate (e.g., NK cells, NKT cells, macrophages and myelomonocytic cells) and adaptive (e.g., Th1 cells, CTL and B cells) immune cells can secrete this cytokine. The Th2-associated cytokine IL-4, together with IL-10, transforming growth factor-β (TGF-β), and glucocorticoid down-regulate the production of IFN-γ [64].

IFN-γ is a major product of fully differentiated Th1 cells and plays an important part in the establishment of an antiviral state, promoting cytotoxicity via both direct and indirect mechanisms [65]. This mediator can also potentiate peptide-specific activation of
CD4+ T cells among APCs via upregulation of the class II antigen-presenting pathway [66].

**IL-4**

IL-4, a 15-KDa monomer produced by Th2 cells, basophils, mast cells, and eosinophils, is involved in allergic reactions and the protective immune response against helminthes and other extracellular parasites [67,68]. IL-4 is the major stimulus for Th2 cell development; suppresses Th1 cell development; and induces IgE class-switching in B cells.

**3. Liver Immunology**

The liver plays a key role in metabolic homeostasis, being responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins [69]. It is also the primary detoxifying organ of the body, removing waste and xenobiotics by metabolic conversion and urinary and biliary excretion. The cell types in the liver that carry out most of these functions are the parenchymal cells, or hepatocytes, which constitute approximately 70% of the cells in this organ. The other 30% are hepatic sinusoidal endothelial cells (LSEC), innate immune cells (antigen-presenting Kupffer cell and dendritic cells), NK cells, NKT cells and adaptive immune cells (T and B lymphocytes) (Figure 3) [73]. The mean yield of intrahepatic lymphocytes (IHL) from humans and mice is similar (10^6 IHL/mg) and indicates that the human liver contains 10^9-10^10 lymphocytes [70,71]. The antigen-presenting cells in the liver are believed to be crucial for the maintenance of tolerance under non-inflammatory conditions [72,73].

About 30% of the blood volume passes through the liver every minute and such tolerance toward endogenous antigens delivered by the blood and the ability to launch immune responses against pathogens are required simultaneously by this organ. This balance is made possible by complex interactions between hepatocytes, antigen-presenting cells and effector cells of the innate and adaptive immune system.
Figure 3. Overview of the intrahepatic immune system (adapted from [73]).

**Kupffer cells**

The Kupffer cells comprise 20% of the non-parenchymal cells in the liver and in fact, represent the largest pool of macrophages in the human body [74]. These cells are located within the sinusoidal vascular space in the periportal area, where they clear endotoxins from the passing blood and phagocytize debris and microorganisms. Kupffer cells can migrate through the hepatic sinusoid and space of Dissé and in this manner establish close contact with passing lymphocytes and hepatocytes, respectively [73]. These cells produce a number of regulatory compounds, including nitric oxide, TNF-α, IL-6, TGF-α and –β, and ROS [75].

**Liver sinusoidal endothelial cells (LSEC)**

Constituting about 50% of the non-parenchymal cells in the liver, LSECs are located between the hepatocytes and the blood flow [74]. LSECs express molecules that participate in antigen presentation, e.g., class I and II MHC and cell surface markers such as CD40, CD80 and CD86 [76,77]. In a manner similar to DCs (see below), LSECs take up antigens via receptor-mediated endocytosis and/or phagocytosis [78].
**Resident hepatic dendritic cells**

With their capacity to migrate and perform immunosurveillance, DCs are the most highly specialized antigen-presenting cells in the liver [79]. In a healthy liver, the predominantly immature DCs are prepared to capture and process antigens [72]. After activation upon encountering an antigen, they migrate from the space of Dissé to the regional lymph nodes, where they present the processed antigen to T lymphocytes [80].

**Lymphocytes**

The lymphocytes scattered throughout the parenchyma and portal tract of the liver contain both conventional and unconventional subpopulations (Figure 4) [73]. The conventional T cells include CD8⁺ and CD4⁺ T-cells. More CD8⁺ than CD4⁺ cells are usually present in the liver, whereas the number of the former is higher in the circulation [73].

The unconventional hepatic T cells include two different populations, i.e., those that express NK markers (called NKT cells) and those that do not. T cells that express the T cell receptor (TCR) γδ belong to this later group and represent 15-25 % of all intrahepatic T cells, thereby constituting the largest population of γδT cells in the body [73].

Accounting for as much as 30 % of the intrahepatic lymphocytes, classical NKT cells are also more abundant in the liver than in other organs [73]. Lipid and glycolipid components of mycobacterial cell walls, i.e., non-peptide antigen targets, are recognized by these cells, which are overwhelmingly CD4⁺ or CD4⁺/CD8⁻-double negative. The dual role of NKT cells involves participation in immune responses against tumor cells and infections and maintenance of tolerance [72,73].

Non-classical NKT cells constitute 30 % of the intrahepatic lymphoid population and are thereby, once again, more abundant in the liver than in any other organ. Their migration to and/or expansion in the liver is controlled by the hepatic NK cells. NKT cells exhibit potent cytotoxic activity against virus-infected and tumor cells and express receptors that both stimulate and inhibit the activities of NK cells [73].
3.1 Innate immune responses in the liver

As mentioned above, the liver is enriched in macrophages (Kupffer cells), NK cells and NKT cells, key components of the innate immune system. Activation of Kupffer cells is triggered by bacterial stimuli, such as LPS and bacterial superantigens. The cytokines produced by these cells play, in turns, roles in modulating the differentiation and proliferation of other cells. For instance, in response to physiological concentrations of LPS, Kupffer cells produce TNF-α and IL-10, which down-regulate receptor-mediated antigen uptake and the expression of class II MHC on LSEC and DCs, thereby reducing T cell activation [74,81]. Kupffer cells are also known to play important roles in connection with resistance to primary and secondary infections. The IL-12 and IL-18 they release regulate NK cell differentiation. Other cytokines, e.g., IL-1β, IL-6, TNF-α and leukotrienes, derived from Kupffer cells promote the infiltration and antimicrobial activity of neutrophils [75].

Upon activation, hepatic NK cells modulate liver injury by balancing local production of pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines through their activating and inhibitory receptors. The inhibitory signals are dominant, i.e., NK cell activation occurs in the absence of ligands, such as class I MHC, which bind to these inhibitory receptors [82]. Such activation leads to rapid production of IFN-γ, which
stimulates hepatocytes and LSEC to secrete the chemokine CXCL9 and thereby recruit T cells to the liver [75].

NKT cells are restricted by CD1, which can be expressed by hepatocytes, as well as by other antigen-presenting cells such as macrophages, DCs and B cells [83]. The majority of classical NKT cells are activated by IL-12, which is produced by DCs and Kupffer cells, and this activation typically results in Fas-dependent cell lysis [84]. NKT cells are also involved in combating both viral and bacterial infections in the liver [85,86].

3.2 Adaptive immune responses in the liver

B cell responses

The function of hepatic B cells remains relatively obscure, since the number of these cells in a healthy liver is low and they have been difficult to isolate. When infected with hepatitis C virus (HCV), the liver behaves as an ectopic lymphoid organ, promoting the proliferation and differentiation of B cells into antibody-secreting cells within the germinal centers of intraportal lymphoid follicles [87].

T cell responses

Upon exposure to LSEC-mediated antigen presentation under non-inflammatory conditions the CD4+ T-cells in the liver participate in hepatic tolerance by secreting IL-4 and IL-10 [88]. An elevated hepatic level of IL-10, which is also produced by CD8+ and Kupffer cells, alters the chemokine receptors on the dendritic cells, thereby attenuating migration of these cells to the draining lymph nodes [89].

The immune response to most liver-tropic pathogens involves a strong and sustained response by CD4+ and CD8+ T cells. In the case of infection with intracellular pathogens presented predominantly on MHC class I molecules, the effector functions of intrahepatic CD8+ T, including the production of Th1 cytokines such as TNF-α and INF-γ and cytolytic mechanisms, have received special attention. The cytotoxic activity of CD8+ T cells is mediated by perforin and granzymes [90,91]. CD8+ T cells proliferate
only in response to recognition of their antigen, which occurs directly in the liver. Accumulation of cells undergoing activation-induced cell death (AICD), in particular CD8+ T-cells, occurs in the liver, indicating that this organ is a site for removal of T cells [92,93].
4. The perfluoroalkyl acids PFOA and PFOS

Numerous xenobiotics disrupt the mammalian immune system and, here, we have focused on the possible immunotoxic effects of perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) (Figure 5) [94]. These compounds belong to the family of perfluoroalkyl acids (PFAA), with a backbone typically 4-14 carbon atoms in length and a charged functional moiety (usually carboxylate, sulfonate or phosphonate). The two most extensively studied PFAAs, PFOA and PFOS, each contain eight carbon atoms [95]. PFAAs are relatively contemporary chemicals, being used only during the past half century [96] and, despite early indications to the contrary, they were long considered to be biologically inactive. Today, much research is focused on understanding the distribution of these compounds in the environment, wildlife, and humans and their potential deleterious effects on health.

![Molecular structure of PFOA and PFOS](image)

**Figure 5.** Molecular structure of PFOA and PFOS (adapted from [94]).

4.1 Physical properties

Covalent carbon-fluorine bonds are among the strongest known. Consequently, compounds containing perfluorinated hydrocarbon backbones are often stable in air at high temperatures (in excess of 150° C); nonflammable; resistant to degradation by strong acids, alkalis, or oxidizing agents; and not subject to photolysis. The stability of these
chemicals renders them practically non-biodegradable and persistent in the environment [97]. The amphiphilic nature of the PFAAs, with their carbon-fluorine chain imparting resistance to oil/water and a somewhat hydrophilic acidic head group, probably explains the extremely low surface tension they provide to solutions.

4.2 Usages

The surfactant properties of PFAAs are ideal for many commercial purposes and the eight-carbon compounds are among the most effective in this context [98]. As fluoropolymers, PFOA and PFOS have found more than 200 industrial and consumer applications, ranging from water-, soil-, and stain-resistant coating for clothing fabrics, leather, upholstery, and carpets, to oil-resistant coatings for paper products approved for food contact, electroplating, electronic etching, bath surfactants, photographic emulsifiers, aviation hydraulic fluids, fire-fighting foams (where the monomers are actually used), paints, adhesives, waxes, and polishes [99].

4.3 Global production

In the year 2000 the production of PFOS and PFOA were 3500 and 500 metric tons, respectively. After the major manufacturer of PFOS, 3M Company, phased out production in 2002, global production of this chemical dropped precipitously to 175 metric tons by 2003 [100]. In contrast, global production of PFOA rose to 1200 metric tons annually by 2004. The consumption of PFOS in all European Union Countries in 2005 was estimated to be 12.23 tons. Recent data show that China and Japan are presently the largest producers of PFOS (Figure 6) [101]. In 2006 the U.S. Environmental Protection Agency initiated a program to eliminate emission and use of PFOA by 2015 [102]. At the same time, other PFAA products are being developed to fill the resulting commercial void.
4.4 Distribution in the environment

In certain Nordic countries, the Netherlands, Spain, Austria, USA and Japan, PFOA and PFOS have been detected in soils, sediment, waste water and sludge, close to as well as far from fluorochemical manufacturing plants [103-108]. As recently documented [109,110] the highest levels of PFAAs are present in the liver of fish-eating animals, including mink, bottle-nose dolphins, polar bears, and ringed seals, living close to industrial areas [111,112].

4.5 Levels in humans

PFOA and PFOS have been detected in human blood (whole blood, plasma and serum), breast milk, liver, seminal plasma and umbilical cord blood [113-118] collected in countries around the globe, including the United States, Canada, Peru, Colombia, Brazil, Italy, Poland, Germany, Belgium, Sweden, India, Malaysia, Korea, China, Japan and Australia. In most cases, occupationally exposed workers and general populations exhibit serum levels of both PFOA and PFOS of approximately 1-2 ppm and
0.01 ppm, respectively. The major routes of human exposure to PFAAs include drinking water, domestic dust, food packaging and cookware [119-124].

4.6 Toxic effects

The toxicology of PFOA and PFOS has been extensively reviewed [100,125-127]. Repeat-high-dose studies with PFOS in rodents and non-human primates have revealed reduced body weight, increased liver weight, lowered blood level of cholesterol, and a steep dose-response curve for mortality [128-130]. A 2-year bioassay, in which Sprague-Dawley rats were exposed to the high dose of 20 ppm PFOS in their diet, showed an increase in hepatocellular adenomas [100,131].

Hepatotoxicity

As noted above, PFOA and PFOS have been associated with hepatomegaly in rodents (Figure 7) and non-human primates, as well as hepatocellular adenomas, testicular Leydig cell and pancreatic acinar cell adenomas following two-year exposure of male, but not female mice to PFOA [132]. Activation of the peroxisomes proliferator-activated receptor-alpha (PPARα) has been suggested to be involved in tumorigenesis (primarily in the liver) induced in rodents by a number of non-genotoxic carcinogens. In rats and mice PFOA and PFOS cause peroxisome proliferation [133-136] and induce peroxisomal fatty acid β-oxidation, enhancing the expression of lipid-metabolizing enzymes and transport proteins [137-140].

Figure 7. Liver hypertrophy in mice after dietary treatment with PFOA and PFOS.
Developmental toxicity

Exposure to PFOA or PFOS in utero delays the development and reduces postnatal survival and growth of rodent pups. Moreover, exposure of rats to PFOS during the last 4 days of gestation is sufficient to reduce neonatal survival [126]. When rodent dams are exposed to PFOS throughout pregnancy, dose-dependent (1-10 mg/kg) deleterious effects are seen in the neonates [141]. All rat pups are born alive, pink, and active with the lowest dose, but with 5 and 10 mg/kg, the neonates become pale, inactive, and moribund within 30–60 min, and all die soon afterwards.

A similar result was reported by Butenhoff [142], Luebker [143] and their coworkers for the highest doses of PFOS (10 mg/kg) and PFOA (30 mg/kg) employed in a two-generation study on rats. Survival improves with lower exposure, being approximately 50 % with 3 mg/kg. Accordingly, the morbidity and mortality of the newborn rats appears to be correlated to their body burden of the fluorochemical (108 ppm PFOS in the serum with the 5 mg/kg dose). Furthermore, morphometric alterations in the lungs of neonates exposed to PFOS were suggestive of immaturity, but the failure of rescue agents and presence of normal pulmonary surfactant indicate that the labored respiration and mortality observed in these pups was not due to lung immaturity [144,145].

Immunotoxicity

Yang and coworkers [146-148] reported on the immunotoxic potential of high-dose short-term treatment of mice with PFOA. When given in the diet to male C57Bl/6 mice for 7-10 days, these fluorochemicals reduce body weight and the size of the epididymal fat pads, elevate liver weight, induce peroxisome proliferation and cause atrophy of the thymus and spleen (Figure 8). The numbers of thymocytes and splenocytes are decreased 90 % and 50 %, respectively, probably due to an inhibition of cell proliferation in the thymus.

In addition to altering splenic cell numbers, these compounds are immunosuppressive in both in vivo and in vitro systems upon high-dose exposure (40 and 30 mg/kg respectively) [146,149]. Even a very low, environmentally relevant dose (5 mg/kg) of PFOS has been reported to suppress humoral immunity [150]. In
addition, Fairely and colleagues [151] demonstrated an elevation in circulating levels of IgE when PFOA (dermal exposure) and ovalbumin were co-administered to rats.

Recently we have demonstrated that PFOA or PFOS alters hematopoietic cell development in the bone marrow, where all the immune cells are generated. Both fluorochemicals reduced the total numbers of different hematopoietic cells belonging to the lymphoid, myeloid and erythroid lineages in the BM in a dose-dependent manner. Thus, the immunosuppressive effects of high doses of these fluorinated compounds are due to impairment of expansion of immune cells at this location. This finding could also explain the reduced populations of neutrophils and macrophages observed in the periphery and tissues, respectively.

**Effects on hormonal levels**

Most recently, several studies have documented depression of serum levels of thyroxine (T4) and triiodothyronine (T3) in rats (adults, adults during pregnancy and neonates) exposed to PFOS, although a corresponding elevation in the level of thyroid-stimulating hormone (TSH) expected to occur through feedback stimulation of the hypothalamus pituitary (HPT) axis is absent [131,141,143,152]. The mechanism behind the hypothyroxinemia observed appears to involve enhanced expression of OATP2 (organic anion transporter protein) and MRP2 (multidrug resistance-associated protein) as a consequence of exposure to PFOS, followed by enhanced uptake and metabolism of thyroxine. Moreover, several investigations [153-155] have reported that administration of PFOA to adult male rats for 14 days reduces serum and testicular levels of testosterone, while enhancing serum levels of estradiol.
5. Peroxisomes

Peroxisomes are single-membrane cytoplasmic organelles present in all eukaryotic cells. They are about one-fifth the size of mitochondria and most abundant in cells of the liver and kidney [156]. These organelles contain more than 60 proteins, including a wide range of oxidases such as urate oxidate, D-amino acid oxidase, polyamine oxidase and many more. They are responsible for a variety of metabolic functions, including β-oxidation of fatty-acids, glycogenesis, catabolism of polyamines and cellular respiration [157-159].

5.1 Peroxisome proliferators

A group of xenobiotics referred to as peroxisome proliferators (PPs), including drugs (hypolipidemic, analgesics, uricosuric), plasticizers, environmental pollutants and industrial chemicals cause substantial increases in the numbers of hepatic peroxisomes together with liver enlargement, particularly in rodents [158]. This phenomenon is associated with enhanced expression of peroxisomal proteins involved in the oxidation of fatty acids and other aspects of lipid metabolism [160] and the underlying mechanism involves so-called peroxisome proliferators-activated receptors. PPs are recognized as non-genotoxic carcinogens.

5.2 Peroxisomes proliferator-activated receptors (PPARs)

In the 1990’s the first nuclear receptor activated by peroxisome proliferators was cloned and designated as the peroxisome proliferator-activated receptor (PPAR) [160]. The three isoforms of PPARs known at present are PPARα, PPARβ/δ, PPARγ. These are encoded by different genes, expressed differentially in various tissues and have specific functions [161] (Figure 9). Three related PPAR isotypes have been identified in vertebrates, including Xenopus, mouse, rat, hamster, and human [162].
5.3 Gene activation by PPARs

The PPARs belong to the family of steroid-retinoid nuclear receptors that act as ligand-activated transcription factors [163]. These receptors form heterodimers with the retinoic X receptors (RXRs) and the resulting PPAR-RXR heterodimer binds to DNA at a so called DR-1 motif or peroxisome proliferator responsive element (PPREs) with the consensus repetitive sequence AGGTCA [160]. In the absence of ligand, the dimer recruits co-repressors, histone deacetylases and chromatin-modifying enzymes, which results in active repression or silencing of transcription [161]. Ligand binding induces conformational changes in the PPAR-RXR complex, which leads to release of repressors and the recruitment of coactivators. These ligand-activated complexes then recruit the basal transcription machinery, resulting in enhanced gene expression.

PPARs bind components of dietary fat or intracellular lipid metabolites with relatively low affinity. In their role as lipid sensors, PPARs regulate lipid homeostasis through the activation of genes whose products are involved in lipid metabolism, storage and transport. PPARs are also involved in the release of anti-inflammatory factors [160,163].
PPAR\(\alpha\)

PPAR\(\alpha\) is expressed at highest levels in metabolically active tissues such as the liver, heart, kidney, skeletal muscle, brown adipose tissue, and spleen [164]. This receptor is involved in hepatic, skeletal and cardiac lipid metabolism, cellular fatty acid uptake, triglyceride (TG) catabolism and glucose homeostasis, as well as exerting anti-inflammatory effects. Furthermore, PPAR\(\alpha\) plays a regulatory role in controlling cardiac metabolic switches and is thus able to prevent cardiac dysfunction. Upon ligand binding, PPAR\(\alpha\) can also act as an anti-atherogenic factor by modulating systemic and local inflammatory responses [164]. The endogenous ligands for PPAR\(\alpha\) include mono-and poly-unsaturated fatty acids, eicosanoids, long-chain fatty-acyl CoAs and certain saturated fatty acids [165,166].

PPAR\(\gamma\)

PPAR\(\gamma\) is expressed at high levels in white and brown adipose tissues, the large intestine and spleen. This receptor plays important roles in adipogenesis, glucose homeostasis, and insulin sensitivity [167]. Activation of PPAR\(\gamma\) leads to up-regulation of fatty acid-metabolizing proteins, which in turn results in adipocyte differentiation. Agonists of PPAR\(\gamma\) have been shown to induce terminal differentiation of normal preadipocytes and liposarcoma cells in vitro and have been proposed for use as potential therapeutic agents against liposarcoma [168].

Little is presently known concerning endogenous ligands for PPAR\(\gamma\). Metabolites of prostaglandin D2 and cyclopentanone prostaglandin PGA1 have been suggested to be potent activators, whereas rosiglitazone and pioglitazone act as synthetic ligands for this isoform [162,169]. There is also evidence that thiazolidinediones (TZDs), a novel class of insulin-sensitizing drugs used in anti-diabetic therapy, activate PPAR\(\gamma\) as well [170].

PPAR\(\beta/\delta\)

PPAR\(\delta\) is ubiquitously expressed in mammalian tissues and was initially believed to have a general house-keeping role [171]. However, it has now been shown that PPAR\(\delta\) knock-out mice exhibit altered dermal inflammatory responses, impaired wound healing,
defects in myelination and embryonal lethality due to placental defects [172-174]. Prostaglandin A and other eicosanoids are ligands for PPARδ [165].

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5.4 PPARs and effects on the liver

PPARα mediates many of the hepatic responses to peroxisome proliferators including peroxisome proliferation and up-regulation of genes encoding fatty acid oxidation systems in the mitochondria, peroxisomes and endoplasmic reticulum (ER). The increased rate of peroxisomal fatty acid β-oxidation increases cellular levels of H₂O₂, which may result in DNA damage and hepatocyte proliferation and thereby contribute to the development of liver tumors observed when rodents are treated with PP [175]. Targeted disruption of the PPARα gene in mice has confirmed the involvement of this nuclear receptor in peroxisome proliferator-induced pleiotropic responses, including hepatocellular carcinoma [176,177].

5.5 The role of PPARs in immunity and inflammation

PPARs are expressed at high levels in different immune cells. PPARα is expressed at higher levels in macrophages, where it controls the efflux of cholesterol [178]. Agonists of PPARα exert anti-inflammatory effects in vascular cells by inhibiting expression of adhesion molecules and enhancing expression of endothelial nitric oxide synthetase (eNOS). However, certain in vivo investigations have found proinflammatory effects of PPARα ligands during endotoximia [179]. PPARs also play a variety of
regulatory roles in connection with the innate immune system, e.g., enhancing removal of apoptotic cells by phagocytosis and regulating the production of antimicrobial peptides [180].

PPARγ is expressed in monocytes, macrophages, DCs, NK cells and T cells. In macrophages, this receptor plays an important role in lipid homeostasis. Agonists of PPARγ also exert anti-inflammatory effects in different model systems, where these compounds inhibit the up-regulation of inflammatory genes by LPS, IL-1β and IFN-γ [181,182]. This receptor is also involved in promoting apoptosis of lymphocytes through agonist-induced activation of T cells and, furthermore, activation of PPARγ appears to inhibit the expression of IL-2, thereby suppressing the development of an immune response [183].

PPARδ is expressed in myeloid cells and its expression is induced during differentiation of human macrophages. Upon activation by its ligands, this receptor can potentially increase triglyceride accumulation in macrophages [184]. PPARβ/δ has also been reported to regulate the production of TNF-α and IFN-γ and is thereby suggested to be involved in wound healing [172].
6. Present investigations

The work presented in this thesis was designed to characterize how the murine immune system is influenced by exposure to PFOA and PFOS.

6.1 Specific aims

**Paper I.** To examine the effects of 0.02 % (w/w) PFOA or PFOS in the diet on the innate immune system of male C57BL/6 mice.

**Paper II.** To characterize the effects of 10-day dietary treatment with different doses (1–0.001 %, w/w) of PFOS on the adaptive immune system of mice and also to examine the possible involvement of PPARα in these phenomena.

**Paper III.** To determine whether sub-chronic (28-day) dietary exposure to an environmentally relevant dose of PFOS (250 μg/kg body weight per day) is immunotoxic toward male B6C3F1 mice.

**Paper IV.** To develop a simple and more effective procedure for the isolation of intrahepatic immune cells from mouse liver based on mechanical disruption.

**Paper V.** To characterize the effects of 0.002 % PFOA or 0.005 % PFOS (sub-immunotoxic doses) on the histology and immune status of the murine liver.
7. Methodological considerations

The Materials and Methods employed in Papers I-V are described in detail in the attached articles themselves. Below some key aspects of the methodology are considered.

7.1 Animals

Throughout our study we used the mouse as our experimental animal, because of the paradigmatic use of this rodent species in immunological research. The murine immune system is quite similar to that of humans and, therefore, the reaction of mice to environmental factors can provide valuable information concerning potential immunotoxic risk to humans. Moreover, mice are small, inexpensive, and easily maintained and reproduce rapidly. The mouse strains we used here were; C57Bl/6, 129/Sv (wild type and PPARα knock-out) and B6C3F1. (Papers I-V)

7.2 High performance liquid chromatography- mass spectroscopy-mass spectroscopy (HPLC-MS-MS)

Our collaborators at the 3M Company (St Paul, Minnesota, USA) have developed a highly sensitive, selective and reproducible HPLC-MS-MS procedure for determination of PFOA and PFOS in complex chemical mixtures. (Papers I, III and V)

7.3 Flowcytometry

This technique enables reliable identification and quantification of small numbers of cells (1 in 10000) of a given phenotype among large mixed populations. (Papers I-V)

7.4 In vitro cell culture and stimulation

The cultures of peritoneal, bone marrow, spleen and liver-derived cells were established to measure the production of various cytokines and antibodies in RPMI-1640 or D-MEM. (Papers I, III-V)

RPMI-1640 contains a bicarbonate buffering system, together with amino acids and vitamins, and is commonly employed to support the growth of both normal and neoplastic leukocytes.
7.5 Assay of cell proliferation

The MTT (methylthiazol tetrazolium) assay is based on the conversion of the yellow tetrazolium salt-MTT to purple-formazan crystals by mitochondria and is routinely used as a quantitative indicator of metabolically active cells. (Paper IV)

7.6 Propidium Iodide (PI) and the Annexin-V assay

PI staining solution is routinely used to assess plasma membrane (PM) integrity in the Annexin V assay. PI stains DNA, in cells that have lost their PM integrity and become permeable to PI as a consequence of either apoptosis or necrosis. (Paper IV)
8. Results

Paper I: High-dose, short-term exposure of mice to perfluorooctanesulfonate (PFOS) or perfluorooctanoate (PFOA) affects the number of circulating neutrophils differently, but enhances the inflammatory responses of macrophages to lipopolysaccharide (LPS) in a similar fashion

After characterizing the effects of high-dose, short-term dietary exposure of mice to PFOS or PFOA on adaptive immunity, we examined a number of parameters associated with the innate immune system of mice, including activation by bacterial LPS after dietary treatment with 0.02% PFOS or PFOA for 10 days.

A first indicator of possible effects on the innate immune system, were the reductions in the total numbers of circulating WBC (leukopenia) and lymphocytes (lymphopenia), following exposure to either compound, whereas the number of circulating neutrophils was decreased only by PFOA. Neutrophils are key cellular mediators of innate immune responses and these findings thus provide an initial indication that at least PFOA may exert an adverse effect on cells in the bone marrow.

The total numbers and relative proportions (percentage) of macrophages in three immune compartments, i.e., the peritoneal cavity, bone marrow and spleen, are influenced differently by exposure to PFOA or PFOS. Both of these perfluorochemicals elevated the proportion, but not the total number of macrophages (CD11b⁺) among the cells isolated from the peritoneal cavity; reduced both the proportion and total number of these cells in the bone marrow; but had no significant effect on the size of the splenic population of macrophages, despite the fact that the overall cellularity of the spleen was strikingly reduced. Thus, it appears possible that bone marrow macrophages are susceptible to, e.g., induction of necrosis and/or apoptosis by PFOA or PFOS, whereas the resident macrophages in the peritoneal cavity and spleen are resistant.

To examine the possible influence of PFOS and/or PFOA on the functional properties of peritoneal, bone marrow and splenic macrophages, we determined the ability of these cells to produce TNF-α and IL-6 ex vivo with and without stimulation by LPS (100 ng/ml). In the absence of this potent activator of macrophages, peritoneal cells isolated from mice treated with either compound produced significantly enhanced levels
of both of these cytokines. The bone marrow cells from animals administered PFOA secreted elevated levels of TNF-α whereas the production of this latter cytokine by spleen cells is reduced by both PFOS and PFOA. *In vivo* exposure to PFOS potently enhances the production of both TNF-α and IL-6 by peritoneal and bone marrow cells after *in vitro* LPS stimulation while, at the same time, attenuating the corresponding production of TNF-α by spleen cells.

A similar pattern is observed following treatment with PFOA, except that in this case, the *ex vivo* production of TNF-α and IL-6 by spleen cells in response to *in vitro* stimulation with LPS was also significantly enhanced. Since macrophages are the major producers of these cytokines in these three compartments, we conclude that exposure to either PFOS or PFOA alters the functionality of these cells. Together, these findings suggest that PFOS and PFOA may sensitize macrophages to external insults, in particular by infectious agents.

The observations documented above raise the question as to whether exposure to PFOS and/or PFOA renders mice more susceptible to inflammatory responses associated with infection. In essential agreement with our *in vitro* findings, pretreatment with PFOS or PFOA prior to the challenge with LPS further potentiates production of these cytokines by both peritoneal and bone marrow cells, while attenuating the *ex vivo* responses of splenocytes to LPS-induced inflammation. Potentiation of the production of TNF-α and IL-6 in response to LPS by pretreatment with PFOS or PFOA suggests that these compounds might render the animal more susceptible to disease conditions (e.g., systemic autoimmune diseases) in which TNF-α and IL-6 play a pivotal role [185,186].

Finally, we found that dietary exposure of mice to a 20-fold lower dose (i.e., 0.001 %, w/w) of PFOS or PFOA for 10 days did not alter any of the parameters examined here.
Paper II: The atrophy and changes in the cellular compositions of the thymus and spleen observed in mice subjected to short-term exposure to perfluoro-octanesulfonate are high-dose phenomena mediated in part by peroxisome proliferator-activated receptor-alpha (PPARα)

Here, we characterized the effects of 10-day treatment with different dietary doses (1–0.001 %, w/w) of PFOS on the immune system of male C57BL/6 mice and also examined the potential involvement of PPARα in these phenomena.

First, the general toxicity of PFOS was determined by monitoring changes in body weight and other signs of toxicity (e.g., ruffled fur, lethargy, poor grooming or other changes in behavior). Mice receiving dietary concentrations higher than 0.02 % exhibited a pronounced dose- and time-dependent weight loss, which was accompanied by lethargy and poor grooming. The 0.02 % dose did not cause toxicity with respect to behavior or appearance, although a significant reduction in body weight, food consumption, and the weights of the thymus, spleen and epididymal fat pads occurred. In contrast, hepatomegaly was observed at all doses tested. These findings indicate a relatively steep dose–response curve for the other effects monitored here in mice and clearly reveal that liver hypertrophy (hepatomegaly) is the most sensitive endpoint in this context.

To evaluate these phenomena in greater detail, we determined the effects of PFOS at dietary levels which do not induce toxicity (i.e., 0.001, 0.005 and 0.02 %) on the overall cellularity and phenotypically distinct cell populations of the thymus and spleen. The highest of these doses led to a marked reduction in the total numbers of thymocytes and splenocytes (84 % and 43 %, respectively), as well as in all of the individual subpopulations of thymocytes and splenocytes.

Consistent with the results obtained by flow cytometry, the histology of the thymus of mice treated with 0.02 % PFOS differed significantly from that of the control animals. The thymic cortex of exposed animals was smaller and virtually devoid of cells and, moreover, the cortical/medullary junction was not distinguishable, in contrast to the well-organized junction in the control organ. At the same time, with the exception of a moderate reduction in the size of the lymphoid nodules, the atrophy of the spleen caused by this same dose of PFOA or PFOS was not associated with any other obvious structural changes.
Since we have previously found that PPARα plays an important role in PFOA-induced modulation of the murine immune system [148] and both PFOA and PFOS can act as agonists for PPARα [187], we next compared the effects of PFOS on wild-type and PPARα-null 129/Sv mice. Although there was a significant increase in liver weight and a marked reduction in the weight of the spleen in both the wild-type and null animal, the weights of the thymus and epididymal fat pads were reduced in the wild-type mice only. As in C57Bl/6 mice, the total numbers of thymocytes and splenocytes, as well as all subpopulations of thymocytes (33–73 %) and splenocytes (37–41 %) were reduced in the wild-type 129/Sv mice. However, in the PPARα-null animals, the reduction in the total numbers of thymocytes and subpopulations thereof was partially attenuated and the effects on splenocytes largely, if not totally, eliminated. Thus this receptor plays a significant role in the immunotoxic effects of high doses of PFOS.

Paper III

28-Day dietary exposure of mice to a low total dose (7 mg/kg) of perfluorooctanesulfonate (PFOS) alters neither the cellular compositions of the thymus and spleen nor humoral immune responses: Does the route of administration play a pivotal role in PFOS-induced immunotoxicity?

A recent study had concluded that sub-chronic (28-day) exposure of mice by oral gavage to doses of PFOS that result in serum levels comparable to those found in general human populations suppress adaptive immunity [150]. Because of the potential impact of these findings on environmental research and monitoring, we examined whether sub-chronic dietary exposure (a major route of human exposure) to a similarly low-dose of PFOS (250 μg/kg body weight/day) also suppresses adaptive immune responses in male B6C3F1 mice.

At the end of the treatment period, the exposed mice had gained somewhat less weight than the untreated controls and the liver mass was elevated significantly; whereas the masses of the thymus, spleen and epididymal fat pads were not significantly altered. This dietary exposure had no effect on the total numbers of circulating leukocytes or of cells in the thymus and spleen. Nor did this treatment alter the relative numbers of the phenotypically distinct types of cells present in the thymus (i.e., immature CD4−/CD8−
and CD4+/CD8+ lymphocytes, mature Th cells and CTL, NKT and NK cells) or spleen (i.e., B lymphocytes, Th cells and CTL, γδT cells, NKT and NK cells, macrophages, granulocytes and plasma cells).

In order to determine whether such 28-day low-dose exposure to PFOS affects humoral antibody responses to a T cell-dependent or T cell-independent antigen, PFOS-treated and control mice were immunized with sheep red blood cell (SRBC) or TNP-LPS on day 23 of the treatment period and antibody responses to this antigen monitored 5 days later. First, we found no difference in the proportion of plasma (CD138+) cells in the spleen, or in the number of these cells that were secreting anti-SRBC IgM antibodies (employing flow cytometry and the plaque-forming assay), respectively. To be even more certain of these findings, we employed ELISA procedures to measure circulating levels of both IgM and IgG antibodies directed specifically against SRBC and TNP and, once again, found no differences between the two groups. From all of the above findings we conclude that a 28-day exposure of adult male B6C3F1 mice to dietary levels of PFOS that results in serum concentrations (11.6 µg/ml) one or two orders of magnitude higher than those observed in occupationally exposed workers, does not compromise the adaptive immune system and, perhaps, the route of administration may have a considerable impact on the immunotoxic effects exerted by PFOS.

**Paper IV**

**Isolation of murine intrahepatic immune cells employing a modified procedure for mechanical disruption and functional characterization of the B, T and natural killer T cells obtained**

Intrahepatic immune cells (IHIC) are known to play central roles in immunological responses mediated by the liver (see the Introduction above) and isolation and phenotypic characterization of these cells is of considerable importance. Therefore, we developed a simple procedure for the mechanical disruption of mouse liver that allows efficient isolation and phenotypic characterization of IHIC. These cells were compared with the corresponding cells purified from the liver after enzymatic digestion with different concentrations of collagenase and DNase.
The mechanical disruption resulted in appreciably higher cell yields (64%) than those obtained by enzymatic digestion (50%). Phenotypic characterization of the purified IHIC by immunofluorescence staining and flow cytometry revealed that the IHIC isolated following our mechanical disruption were, as expected, heterogeneous in composition, containing both innate and adaptive immune cells of which B, T, NK and NKT cells, granulocytes, macrophages, \( \gamma^\delta \)T and so-called myeloid suppressor cells, were the major populations. The composition of IHIC obtained with enzymatic digestion was significantly different, containing a higher percentage of B cells but, at the same time, considerably lower proportions of NKT and \( \gamma^\delta \)T cells. These findings indicate that murine intrahepatic NKT cells are highly sensitive to treatment with collagenase and DNase.

In order to evaluate whether IHIC obtained with our improved procedure for mechanical disruption were functionally intact, we examined in vitro the proliferative responses of the B, T and NKT cells, as well as the production of IgM by B cells and IFN-\( \gamma \) by T cells and NKT cells upon exposure to their specific activating stimuli. In response to LPS, hepatic B cells proliferated and produced significant amounts of IgM; T cells responded to ConA by producing high levels of IFN-\( \gamma \); and NKT cells responded to \( \alpha \)-GalCer by synthesizing significant amounts of IFN-\( \gamma \). These observations demonstrate that these three cell populations in IHIC isolated following the improved mechanical disruption are functionally immunocompetent.

**Paper V**

**Dietary exposure to perfluorooctanoate or perfluorooctane sulfonate induces hypertrophy in centrilobular hepatocytes and alters the hepatic immune status in mice**

As shown above, as well as by numerous other studies, the liver is the major organ affected by the exposure of rodents to PFOA or PFOS. The hepatic effects of these compounds include pronounced hepatomegaly, with long-term exposure elevating the incidence of liver tumors in the rat [132]. Accordingly, we investigated the effects of these compounds on liver structure and the hepatic immune system.
Dietary treatment of male C57Bl/6 mice with 0.002 % PFOA and 0.005 % PFOS for 10 days exerts minimal or no effect on food intake, body weight gain, the mass of the thymus, spleen and fat pads, serum levels of alanine aminotransferase (ALT) or aspartate aminotransferase (AST), hematocrit, or blood level of hemoglobin. At the same time, PFOA- and PFOS-exposed animals exhibited a significant increase in liver mass (1.6- and 1.4-fold respectively); with a moderate increase in the serum activity of alkaline phosphate (ALP) (1.4-fold) and a reduction in the serum level of cholesterol (by ≥24 %) and triglyceride (>25 %).

Microscopic examination of liver sections exhibited structural alterations, particularly in the parenchymal cells. With both compounds, the hepatocytes surrounding the central vein were hypertrophic and displayed cytoplasmic acidophilic granules.

PFOA was found to induce a pronounced elevation (200 %) in the total numbers of non-parenchymal IHIC. Moreover, the total numbers of phenotypically distinct types of myeloid-, lymphoid- and erythroid-related cells (granulocytes, myeloid suppressor cells, macrophages, Th cells, CTL, NK cells, γδT and erythroid progenitor cells) were elevated. In contrast, exposure to PFOS enhanced only the numbers of erythroid progenitor cells, without affecting other types of IHIC. Following exposure to PFOA or PFOS, hepatic levels of the cytokines TNF-α, IFN-γ and IL-4 were lower than in the livers of control animals. Finally we found that exposure to PFOA or PFOS induced 4 to 5-fold increases in hepatic levels of erythropoietin, together with elevating the numbers of erythroid progenitor cells present. Therefore we could conclude that the alterations in the structure and functions of parenchymal and non-parenchymal cells in the liver upon exposure to PFOA or PFOS might alter the susceptibility of this organ to other toxins, drugs and hepatotropic infectious agents.
9. Concluding remarks

The five major findings documented here are the following:

I. High-dose, short-term exposure to either PFOS or PFOA activates certain components of innate immunity in mice, in contrast to their suppressive effects on adaptive immunity.

II. Short-term immunomodulation caused by PFOS in mice is a high-dose phenomenon, with the thymic changes being partially dependent on the PPARα, and splenic responses largely eliminated in its absence. On the other hand, hepatomegaly is independent of PPARα.

III. Sub-chronic dietary exposure of mice to PFOS for 28 days, resulting in serum levels approximately 8–85-fold greater than those observed in occupationally exposed individuals, does not exert adverse effects on adaptive immunity.

IV. The simple procedure for the mechanical disruption of mouse liver described here results in more efficient isolation of functionally competent IHIC for various types of investigations.

V. In mice, PFOA- and PFOS-induced hepatomegaly is associated with significant alterations in hepatic histophysiology and immune status, as well as induction of hepatic erythropoiesis.
10. General discussion and future perspectives

Upper-bound estimates of serum concentrations in general human populations are 100 and 14 ng/mL for PFOS and PFOA, respectively [188,189]. Because PFOS and PFOA are persistent in the environment [100,190] have long elimination half-lives in humans [191,192], and have been detected in most serum/plasma samples from the general population [188,192,193] it is important to consider the potential relevance of immune changes observed here in mice to the body burden of PFOS and PFOA in the general population. We observed that changes in the immune system occurred only at a dose (0.02 % in the diet) that produced significant reductions in body weight and fat. Because PFOS and PFOA are not metabolized [194], are distributed primarily in the extracellular space [191], and are eliminated slowly in humans [192], serum concentrations can be used as a measure of the body burden [100,193]. These properties allow bridging of differences in the elimination rate between species in connection with comparison of body burdens. As a result, serum concentrations associated with effects and no-effect in experimental toxicological studies on rodents can be compared to those measured in human biomonitoring studies, as a means of evaluating potential health risk.

The findings in Paper I, demonstrate that at least certain perfluorinated compounds can exert adverse effects on the innate branch of the immune system, an important defence system found in all plants and animals. The mechanisms underlying the immunotoxic effects of both PFOS and PFOA on both the adaptive and innate branches of the immune system require further elucidation. Toxicokinetic investigations will help clarify whether these compounds exert their immunotoxic effects directly (e.g., via PPARα or PPARβ activation in primary and/or secondary immune organs) or indirectly (e.g., by being accumulated in the same organs). Investigations of this nature are currently being performed in our laboratory.

We have recently observed that the bone marrow, a primary immune organ, is a major site for accumulation of PFOS in the mouse [195]. Our subsequent investigation on the effects of PFOA and PFOS on hematopoietic cells in the bone marrow of mice revealed that while exposure to low doses of these compounds affects mainly the development of B lymphocytes, dietary administration of high doses exerts adverse effects on the cells of most (if not all) hematopoietic lineages including the lymphoid,
myeloid and erythroid lineages. Since PPARα apparently is not expressed in murine bone marrow cells [196], PFOA and PFOS may interfere with hematopoiesis via a mechanism independent of this receptor. We hypothesize that these perfluorinated compounds hinder the development of immune cells in the BM by disrupting cell-to-cell communication in this organ, which in turn reduces the production of B cells and T cell progenitors. We believe that testing this hypothesis would provide insight into the mechanisms underlying the immunosuppression caused by PFOA and PFOS.

Moreover, Bogdanska and coworkers [195] have reported that PFOS accumulates in the fetal liver, where hematopoiesis occurs prior to birth and, in addition, after birth the pups looked pale, probably because the supply of oxygen was insufficient [80]. It would be extremely interesting to examine the generation of fetal immune cells following treatment of the pregnant dam with these fluorochemicals.

The dietary exposure employed in Paper III would appear to be a more realistic model for human exposure than administration by gavage. At the same time, it should be emphasized that humans and other organisms are exposed to PFOS (and PFOA) for much longer periods than 28 or even 60 days, elimination half-lives being 5.4 and 3.8, respectively. In addition, there may be certain subpopulations of humans and other animals that are particularly susceptible to the immunotoxic effects of this fluorochemical. Obviously, further studies on these issues are required, e.g., exposure of the mice at an environmental dose of PFOA or PFOS for 6 months or 1 year, with subsequent evaluation of their immune status.

In Paper IV, we have developed a simple procedure for mechanical disruption of mouse liver that allows isolation of IHIC in yields considerably higher than those achieved by other reported procedures. In addition, the cells thus isolated are amenable to culturing and are functionally immunocompetent. This procedure should prove to be highly valuable in connection with attempts to elucidate the hepatic mechanisms underlying the development of immunological tolerance, as well as other immunological responses mediated by the liver. Furthermore, it will be of considerable interest to examine changes in the composition and activities of IHIC in response to environmental agents such as pathogens and xenobiotics.
Our findings in Paper V demonstrate that exposure of mice to 0.002 % PFOA or 0.005 % PFOS induces pronounced hypertrophy in centrilobular hepatocytes and alters the hepatic immune status in mice. Further studies should be designed to determine the consequences of the hepatic immune alterations caused by PFOA or PFOS, as well as to elucidate whether these compounds exert their effects directly (e.g., by binding to PPAR\(\alpha\), \(\gamma\) and/or \(\beta\) or the erythropoietin receptor) or indirectly (e.g., by inducing hypoxia in the cells affected, thereby stimulating erythropoietin production). Investigations of this nature are currently being performed in our laboratory.

Finally, detailed immune testing could be performed on occupationally or environmentally exposed human populations e.g., those living in close proximity to fluorochemical manufacturing plants. Although it is now well established that PFOA and PFOS exert suppressive effects on the adaptive immune system in rodents [146,150,197] little or no information is available on the possible influences of these compounds on the human immune system. Evaluation of this issue is of considerable importance in in vitro studies, designed to test for alterations in the functions of both un-stimulated and mitogen-stimulated peripheral blood mononuclear cells (PBMCs) in response to PFOA or PFOS might provide valuable information concerning the effects of these compounds on the human immune system. Furthermore, the specific humoral immune responses following vaccination (e.g., for influenza virus) of occupationally-exposed individuals could be compared to the responses of un-exposed individuals. Finally, elucidating the effects of perfluorinated chemicals in mice reconstituted with human immune cells, so-called SCID-human mouse, might also be highly valuable [198].
11. Acknowledgements

My thesis has finally been completed after invaluable contributions in various ways by several people and dedicated researchers. Especially, I would like to acknowledge:

Joe DePierre, my supervisor, for your excellent scientific guidance and never-failing enthusiasm and support during the last 6 years. Thanks also for always taking the time for inspiring discussions and for giving constructive criticism and making the work stimulating and joyful. We enjoy your songs and the delicious food at your place. Barbara, thanks for your care and your company at the gym.

Manuchehr Abedi-Valugardi, my hands-on advisor, for enthusiastically introducing me into this department. Thanks also for stimulating discussions, planning and for always seeing the bright side of a problem. Your tremendous support and caring have given me strength and confidence throughout the years. I have really enjoyed your positive attitude, which has made the work more fun.

Other members of our group: Buck Nelson for scientific advice and critical reviewing our manuscripts and your intelligent and insightful advice at our weekly group meetings; Stefan Nobel, for your support, encouragement and correction of the Swedish abstract; and Jasna Bogdanska, for all the nice hours we spent together in the lab, for your care and for being such a very nice colleague, and for our enjoyable discussions during the morning coffee break. All of my other co-authors for their important contributions to the different studies.

Stefan Nordlund, for generous support and care during my entire PhD period. Thanks for your valuable comments on my thesis. Thanks to Inger Carlberg for being available to discuss any and all issues in a caring fashion.

Elzbieta Glaser and Åke Wieslander for your intelligent advice and dignified personalities.

John Butenhoff, from the 3M Company, for making this collaboration possible. Thank you for your time, active interest in our work, detailed comments on our manuscripts and valuable suggestions concerning the thesis. Thanks to Dave J Ehresman for analyzing the serum samples.
My dear colleagues at the department: Anki, Ann, Haidi, Maria, Lotta and Lollo for your patient help with administrative things and ‘‘always-finding-a-solution’’; Torbjörn and Peter, for helping me with computer problems; Håkan and Bengt for technical support. Special thanks to Bogos, for sharing your knowledge, for delivering packages and for your friendly manner. You were the first person who helped me find Joe’s office when I was almost lost in this big department. I wish you could have been here to share my joy about completing my PhD thesis.

Eva, Solveig and Ellinor in the animal house, for your kind help in taking care of the mice. Eva, you create a pleasant atmosphere in the animal house and always welcome me there with smile.

The great atmosphere at DBB is created by wonderful people: Candan, Pedro, Tiago, Beata, Catarina, Pilar, Gabriela, Joachim and Martin Ott group for the good times and enjoyable moments. You are always smiling and are nice to talk to. Thank for our non-scientific chats during the lunch and coffee breaks. Salomé, the friendly scientist, your energy will guarantee you a bright future in science. Changron, for helping with the practical aspects of this thesis. I am grateful to both of you for taking the time to help me prepare for the ”big exam”.

My degree project and summer students, you each made tremendous progress during your stay in our lab. I learned so much from you. You have bright futures ahead. I also wish to express my sincere gratitude to all the people in Lappis, my good neighbors Susmita, Shapla, Rekha and your families. I will never forget the pleasant times we spent together, especially the BBQs. Thank you for friendship and for being such a nice group of people. I am grateful to Rekha and Emon for your help with the final preparations required to get this thesis into print. Thanks and good luck with your own PhDs.

Zarina Nahar Kabir, my cousin, we are lucky to have you. Thanks for your care and support. Tonima and Shanta, my niece, for your kind heart and dignified personality. Dr. Atiqul Islam for your support and great sense of humor.

Tamanna, my friend, we had delighted times together when you were in Stockholm.

Khaleda, my sister, thank you for always being there for me. Many thanks for all of your kind help and support at the beginning when I didn’t know where any place in Stockholm was. Your enthusiasm for science has made a lasting impact on me. Your
encouragement has been important. Best wishes in your research and life. Apu, my brother-in-law, for helping to create a stimulating and friendly environment outside the world of science. Thank you for your great laughter. My cutypie nephew, Arian, I feel delightful when you are around. I like your attitude and smile.

My mother, for your never-ending love, raising me with your spirit and encouraging me to reach this level. To my father, I can never find a more trustworthy, lovely and charming person than you in all my life. This thesis is as much yours, mother and father, as it is mine. My brother (and his family), I appreciate so dearly your constant encouragement. Thanks for supporting our parents in this critical situation. I thank my parents-in-law for their affection and constant support.

My dearest husband, Jubayer, for always putting my needs ahead of your own, and for taking care of everything when I am busy. Thanks a lot for editing my thesis and for the invaluable comments.

Above all, my lovely little daughter, Ilma, for being the joy and the meaning of my life. For your big smile and your patience during these years and especially during the final work on this thesis. You have such a nice personality.

These studies were made possible by unrestricted grants from the 3M Company (St Paul, Minnesota, USA).
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