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UNIVERSITÀ DEGLI STUDI DI MILANO

In vitro Evaluation of the Immunotoxic Effects of Perfluorooctanesulfonic acid (PFOS) on the Maturation of Dendritic Cells

In vitro εκτίμηση ανοσολογικών επιπτώσεων του Υπερφθοροοκτανοσουλφονικού οξέος (PFOS) στην ωρίμανση των δενδριτικών κυττάρων

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* Η πτυχιακή αυτή πραγματοποιήθηκε στα πλαίσια του προγράμματος ***Erasmus*** *

ABSTRACT

Οι υπερφθοροαλκυλ- και πολυφθοροαλκυλιωμένες ουσίες (PFAS) είναι μια ομάδα χιλιάδων ανθρωπογενών χημικών ουσιών που χρησιμοποιούνται ευρέως σε πολλά καταναλωτικά και βιομηχανικά προϊόντα, στα οποία εκτίθενται οι άνθρωποι και το περιβάλλον. Προκαλούν αυξανόμενη ανησυχία λόγω της επιμονής τους στο περιβάλλον, της κινητικότητας, της τοξικότητας και της βιοσυσσώρευσης τους. Μελέτες σε ανθρώπους έχουν δείξει ανεπιθύμητες ενέργειες των PFAS στο ανοσοποιητικό σύστημα σε χαμηλά επίπεδα έκθεσης, συμπεριλαμβανομένης της μειωμένης απόκρισης αντισωμάτων κατά του εμβολιασμού. Ο σκοπός αυτής της μελέτης ήταν να διερευνήσει την επίδραση του υπερφθοροοκτανοσουλφονικού οξέος (PFOS) στην ωρίμανση των δενδριτικών κυττάρων (DC). Ως πειραματικό μοντέλο χρησιμοποιήθηκε η ανθρώπινη προμυελοκυτταρική σειρά THP-1. Πειράματα διεξήχθησαν, χρησιμοποιώντας τόσο αρχέγονα όσο και THP-1 διαφοροποιημένα σε ανώριμα δενδριτικά κύτταρα (iDCs) για την αξιολόγηση των επιδράσεων του PFOS στην ωρίμανση των κυττάρων. Για τη διαφοροποίηση των DCs, τα κύτταρα THP-1 υποβλήθηκαν σε επεξεργασία για 5 ημέρες με rhIL-4 και rhGM-CSF για να αποκτήσουν τις ιδιότητες των iDCs. Στη συνέχεια, την 5^{η} ημέρα δημιουργήθηκαν ώριμα DCs (mDCs) από iDCs με προσθήκη ενός κοκτέιλ ωρίμανσης. Ως δείκτες διαφοροποίησης και ωρίμανσης των DCs αξιολογήθηκαν το CD83, CD86 και HLA-DR με ανάλυση κυτταρομετρίας ροής. Τα κύτταρα εκτέθηκαν σε αυξανόμενες συγκεντρώσεις PFOS (0,1–10 mg/ml). Τα αποτελέσματα που παρατηρήθηκαν στα mDCs προτείνουν μια ανοσοκατασταλτική δράση του PFOS, αλλά απαιτούνται επιπλέον πειράματα για να επιβεβαιωθούν. Ωστόσο, τα αποτελέσματα για το PFOS μπορούν να χρησιμοποιηθούν ως σημείο αναφοράς για την παροχή γνώσεων της σχετικής δραστικότητας συγγενικών PFAS, μιας και πρόκειται για ένα από τα πιο ευρέως χρησιμοποιούμενα και μελετημένα χημικά της κατηγορίας αυτής.

1. INTRODUCTION

1.1. Immunotoxicology

1.1.1. Perfluoroalkyl- and polyfluoroalkyl substances (PFAS)

Perfluoroalkyl- and polyfluoroalkyl substances (PFAS) are a large group of thousands of anthropogenic chemicals characterized by a broad range of diverse chemical and physical properties (Ng Carla et al., 2021). In fact, these compounds can be gases, liquids, or solid high-molecular weight polymers and can also be classified as long chain or small chain molecules. Despite the wide variety of PFAS structure and properties, the carbon-fluorine bond, one of the strongest chemical bonds in organic chemistry, is a common feature among them (Cousins et al., 2020). Due to these bonds, PFAS are highly stable, water-repellent and oil-repellent. Moreover, they do not easily degrade naturally and thus accumulate over time or they are converted into highly stable products (Wang Z et al., 2017; Cousins et al., 2020; Glüge et al., 2020; Johnson et al., 2021). This is why they are described as persistent organic pollutants (POPs), sometimes also known as *Forever Chemicals*(Stockholm Convention 2009; Cousins et al., 2020). Overall though, their unique desirable properties make PFAS useful in a variety of consumer products and industrial processes such as food contact materials, leather and textiles, construction, in household products, in fire-fighting foams, in the electronic industry and also in the energy sector (Christopher Lau et al., 2007; Johnson et al., 2021; Rogers et al., 2021). Their extensive use and their environmental persistence increase their presence in water, air and soil and subsequently also in plants, animal tissue and human blood serum (Wang Z et al., 2017; Rogers et al., 2021). Widespread exposure to PFAS in combination with their long biological half-lives led to measurable levels of those chemicals in humans causing cancer, endocrine disruption, developmental and liver toxicity, lipid and insulin dysregulation and immunotoxicity (Brase et al., 2021; Fenton et al., 2021; Rosato et al., 2022).

1.1.2. The immune system

The immune system is a complex and highly regulated network of cells and proteins that uses a complex array of protective mechanisms to control and usually eliminate pathogen organisms and toxins. The balance between the inhibition and activation signals is the key regulatory mechanism of the immune system, as an imbalance may result in

immunosuppression or excessive immunostimulation either of which is detrimental to the overall health of the host (Wu HJ et al., 2012).

The two main categories of the immune system are the non-specific innate system and the adaptive system. The innate system is the body's first line of defense against pathogens and includes physical and biochemical barriers, while the adaptive one involves all types of lymphocytes and can be distinguished into two main defense mechanisms:

- Cell-mediated immunity (mediated by T lymphocytes and directed against intracellular pathogens or virus-infected cells)
- Humoral immunity (mediated by antibodies produced by B lymphocytes and directed at extracellular pathogens)

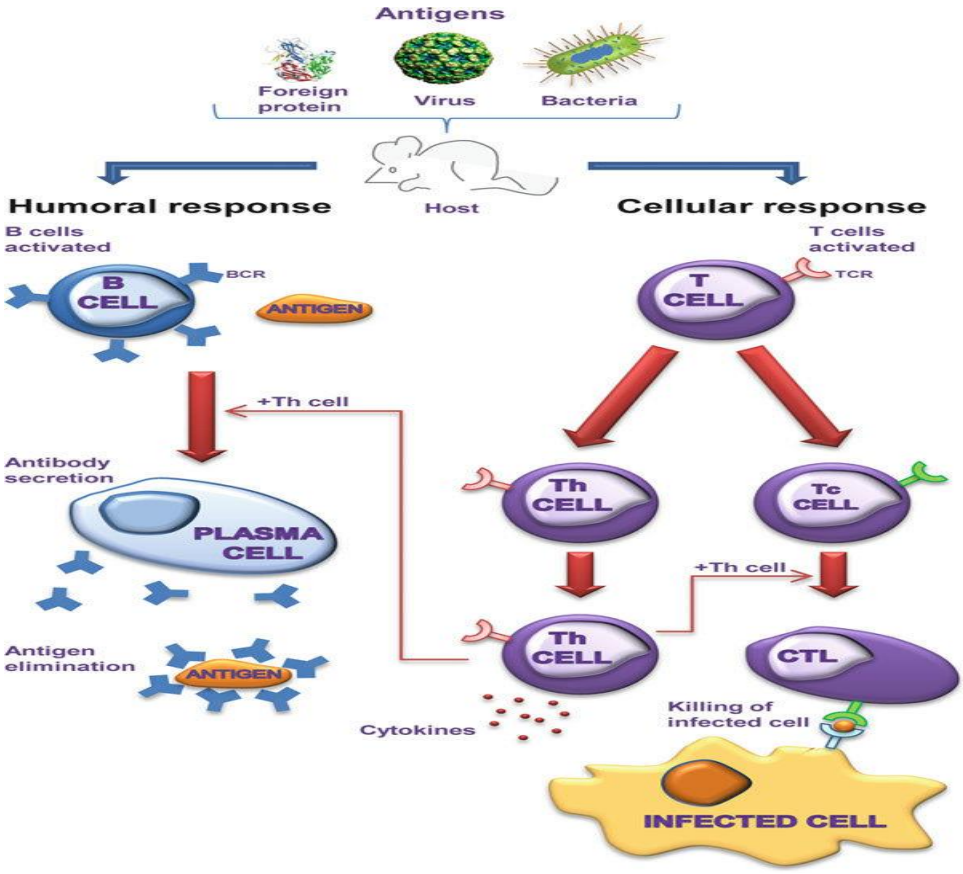


Figure 1.1 – The humoral and cell-mediated branches of the immune response.
[Modified by: Bárcena J et al., 2013]

An intact immune response involves many subsets of leukocytes. Mature, circulating leukocytes differentiate from hematopoietic stem cells. In particular, lymphoid stem cells give rise to B cell, T cell, and natural killer (NK) cell lineages, while myeloid stem cells finally produce neutrophils, monocytes, eosinophils, basophils, mast cells, megakaryocytes, and erythrocytes. Immune cells communicate with each other by directly binding to receptors on

each other's surfaces or through a network of cytokines allowing them to cooperate and finely regulate their activity (Janeway CA et al., 1985).

Leukocytes are the cells that orchestrate immune reactions through various mechanisms, such as the production of antibodies, phagocytosis, antigen presentation for the stimulation of cell-mediated immunity or direct killing of pathogen-infected and tumor cells (Larosa DF., Orange JS., 2008). B cells mature in the bone marrow and their role is to produce and secrete antibodies in response to antigen recognition (J.H. Dean et al., 2008).

NK cells are large granular lymphocytes that have cytotoxic effects on infected cells and do not require any specific antigenic activation (Vivier et al., 2008). T cells mature in the thymus where they are programmed to distinguish between self and non-self antigens (Kaminski et al., 2008). They can be differentiated into cytotoxic T cells or helper T cells (Th). The former are effector cells that target infected or neoplastic cells and eliminate them (Janeway et al., 2001), while the latter have a regulatory role through the release of cytokines. Unlike B cells, T cells require the presence of antigen-presenting cells (APCs) to be activated. Macrophages are phagocytic cells that can act as APCs to T cells and reside in different tissues, including liver (Kupffer cells), brain (microglia) and skin (Langerhans cells) (J.H. Dean et al., 2008). These cells are responsible to expose on their surface the receptor that binds with the major histocompatibility complex (MHC) present on surface of T-cells. Monocytes can differentiate further into macrophages in peripheral tissue compartments and dendritic cells (DCs).

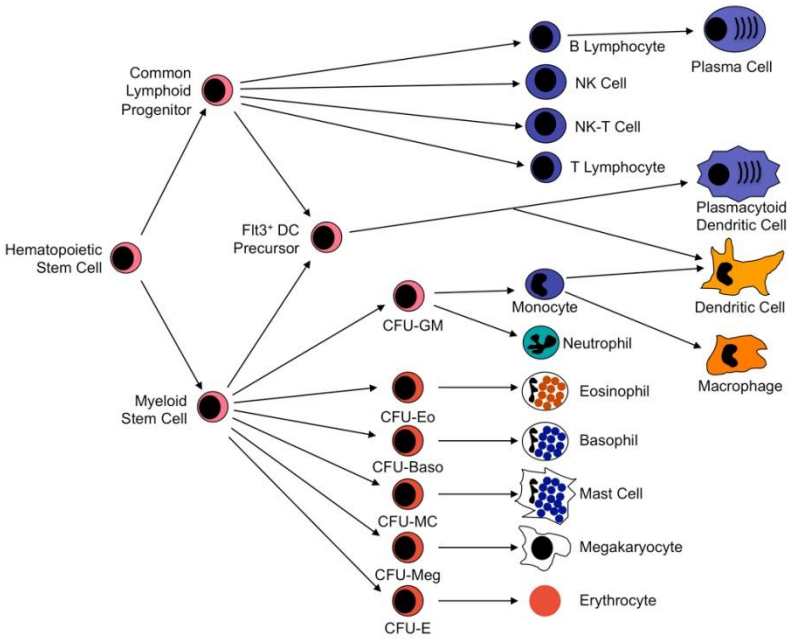


Figure 1.2 – Hematopoietic Stem Cell-Derived Cell Lineages
[Modified by: https://jamanetwork.com/journals/jama/fullarticle/419313]

DCs are specialized myeloid cells generated in the bone marrow. DCs reside predominantly within the lymphoid tissues (spleen, thymus) following migration but can also be found in non-lymphoid tissues (skin, heart, lung) where they develop into their steady state. DCs are the most potent APCs, which play a critical role in the regulation of the adaptive immune response through activation and polarization of naive T cells (Huston DP, 1997; Chaplin DD, 2010).

In their steady state, DCs are able to capture and present antigens to T-cells, but they are not able to initiate an immune response rather than being tolerant (L. Bonifaz et al., 2002). Immature DCs (iDCs) are induced by infectious agents and inflammatory products. At the onset of an inflammatory response, DCs undergo a process of maturation expressing increased levels of cell surface MHC and co-stimulatory molecules such as CD40, CD80, and CD86. In addition, they secrete various cytokines and lead to the binding and activation of T cells in the T-cell areas of lymphoid tissues, thereby inducing immune responses (Banchereau J and Steinman, 1998).

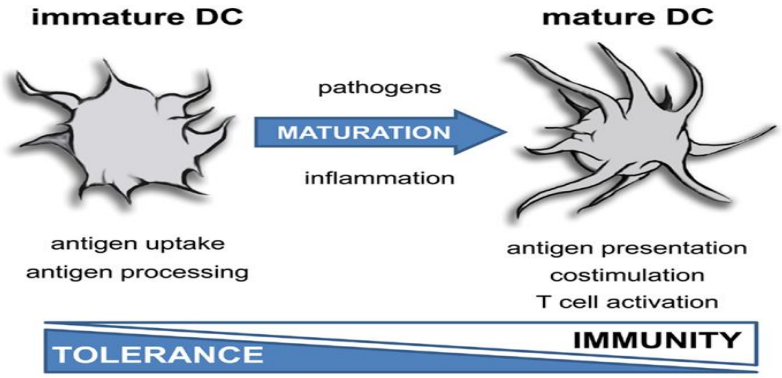


Figure 1.3 – Function of dendritic cells based on maturation
[Modified by: Hubo M et al., 2013]

The presentation of antigens from DCs to T cells requires an active interaction between the two cell types. Mature DCs (mDCs) express a receptor for chemokines, CCR7, which is specific for the recognition of two other chemokines, CCL19 and CCL21 expression on lymphatic endothelium and T cells. This interaction enables the mDCs to migrate towards T cell-rich areas (Quah & O'Neill, 2005). Three signals are required for the complete activation of T lymphocytes as shown in Figure 1.4. The first signal involves the interaction between the T cell receptor (TCR) and the antigen-MHC complex (Corthay, 2006). More in detail, T cell receptors recognize antigens, such as small chemicals, presented by the specific HLA molecule of the MHC on the DCs. Antigens are also described as haptens according to the hapten/pro-hapten concept. Haptens are small molecules (<1000 Dalton), hence; the

immune system cannot recognize them efficiently. Therefore, the parent or reactive metabolites may covalently bind to serum or tissue proteins like albumin to form a complete antigen. These conjugates are the ones loaded onto HLA molecules of the MHC (Aiba S et al., 1997). Then, the CD4 or CD8 molecules bind to the MHC molecule, in order to stabilize the whole structure. The next signal (namely signal two) requires the expression of a co-stimulatory molecule present on T cells named CD28. This molecule binds to one of two molecules on the DCs, CD80 or CD86, and initiates T cell proliferation. The last signal is coordinated by various cytokines that determine and regulate the further conversion of T cells into specific subpopulations which will in turn mediate further immune responses (Corthay, 2006).

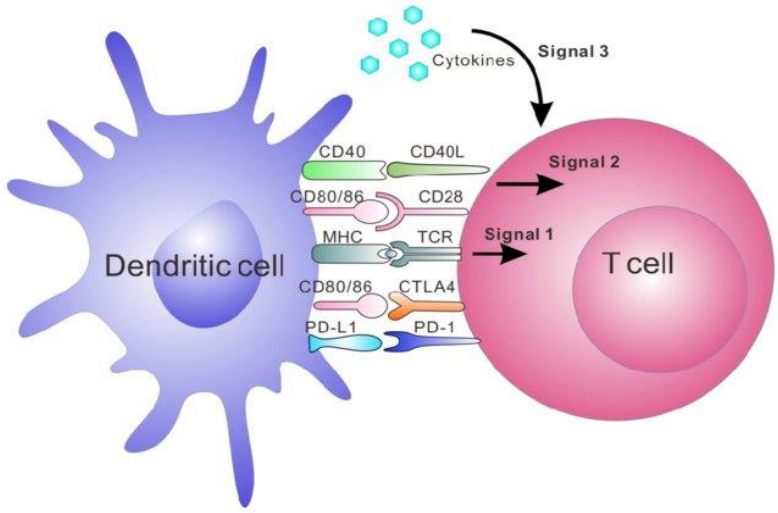


Figure 1.4 – Interaction between DCs and T cell.
[Modified by: Wang C et al., 2017]

According to Gell and Coombs (1968), hypersensitivity reactions can be classified into four types, as also shown in the Figure 1.5 below. They differ by immune reactant, antigen form and mechanism of action and therefore are more difficult to assess (Coombs & Gell, 1968).

- Type I: Immediate type reactions or anaphylactic reactions are usually developed 30 minutes after secondary allergen exposure and are mediated by IgE antibodies against the soluble antigen. On initial exposure, plasma cells are stimulated, by T cells, to produce IgE, which bind to receptors of mast cells and basophils. On re-exposure, mast cells are activated and trigger the release of histamine and other inflammatory factors resulting in reactions such as asthma or systemic anaphylaxis.
- Type II or cytotoxic reactions: In this type, IgM or IgG antibodies are directed against cell surface antigens and induce cellular damage by phagocytosis, complement

system activation, and cell-mediated cytotoxicity. These cytotoxic reactions develop from hours to days and usually result in hemolytic anemia.

- Type III or immune-complex reactions: The immune-complex reactions have a delayed onset and are mediated by the formation of a soluble complex between an antigen and an immunoglobulin (IgM or IgG). Deposition of these complexes in various tissues induces complement activation that leads to the recruitment of inflammatory cells (monocytes and neutrophils), which are responsible for tissue damage.
- Type IV or delayed type: Unlike the other types, this reaction is not antibody-mediated but rather a type of cell-mediated response. The T Cell-Mediated or Delayed Hypersensitivity Reactions typically occur at least 48 to 72 hours and sometimes days to weeks after exposure. The first contact with the antigen is asymptomatic and memory cells are developed. Upon a second contact with the antigen, the already sensitized T lymphocytes release pro-inflammatory cytokines that activate and recall macrophages, which generate an inflammatory response. Despite the different mechanisms involved in the activation of T cells, the end result is the release of cytokine and inflammatory mediators with the subsequent recruitment of inflammatory cells (Dykewicz, 2020). Based on the type of T cell involved and the cytokines produced, Type IV reactions are further subdivided into type IVa (Th1 cells with macrophage activation), IVb (Th2 cells with eosinophils activation), IVc (cytotoxic T-cells), and IVd (neutrophils activation) (Pichler WJ, 2003). Clinical manifestations vary and range from severe cutaneous reactions such as Stevens-Johnson Syndrome (SJS) to liver toxicity (Proksch et al., 2018).

Antibody (I–III) and T cell-orchestrated hypersensitivity reactions (IVa–d)

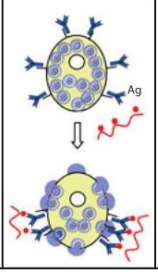
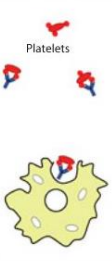
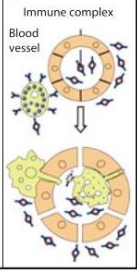
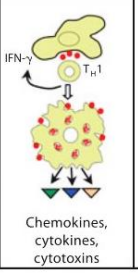
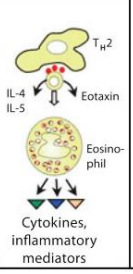
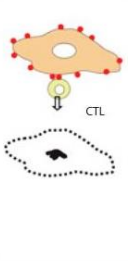
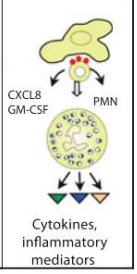
	Type I	Type II	Type III	Type IVa	Type IVb	Type IVc	Type IVd
Immune reactant	IgE	IgG	IgG	IFNγ , TNFα (T_H1 cells)	IL-5, IL-4/IL-13 (T_H2 cells)	Perforin/ granzymeB (CTL)	CXCL-8, IL-17 GM-CSF (T cells)
Effector	Mast cell activation	FcR+ cells (phagocytes, NK cells)	FcR+ cells complement	Macrophage activation	Eosinophils	T cells	Neutrophils
							

Figure 1.5 – The extended Coombs and Gell classification of HDRs.
[Modified by: Hausmann & Schnyder, 2012]

1.1.3. An overview of Immunotoxicity

According to the definition provided by the European Food Safety Authority (EFSA), immunotoxicity is *"any adverse effect on the immune system that results from exposure to toxic substances"* and can be classified as immunosuppression or immune-enhancement (Peyton Myers L., 2018). Immunosuppression is the term used to describe the reduction in the capacity of the immune system to ward off a challenge leading to a reduced response to vaccination or, more severely, a higher susceptibility to pathogens. Immunoenhancement refers to the inappropriate activation of the immune system, which usually results in hypersensitivity responses, such as an allergy, or in autoimmune reactions (DeWitt JC et al., 2019).

1.2. Toxicological Evaluation of PFOS

Taken together, results of epidemiological studies along with toxicological studies provide evidence that perfluorooctane sulfonic acid (PFOS) is an immune hazard to humans. Most of those studies strongly support the idea that PFOS suppresses the antibody response in humans and experimental animals (National Toxicology Program, 2016). PFOS is also suspected to suppress infectious disease resistance and NK cell activity in humans based on moderate level of evidence from animal studies and low or inadequate level of evidence from human studies and also hypersensitivity evidence are reported (National Toxicology Program, 2016).

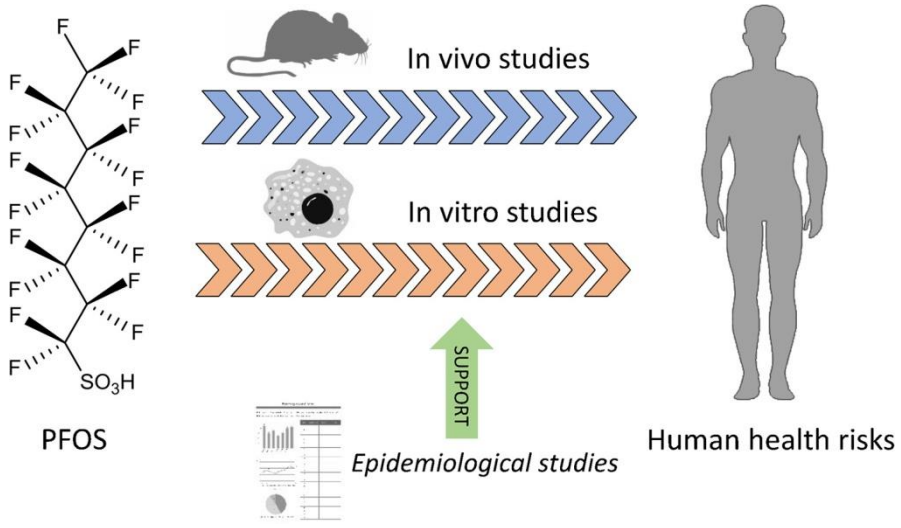


Figure 1.6 – Assessment of the effects of PFOS on humans

[Modified by: Zeng Z et al., 2019]

1.2.1. *In vivo* Methods

Humans

Antibody response refers to antibodies produced towards usually a specific vaccine, which is measured in human populations exposed to an exogenous agent. Antibodies (proteins found in blood and other body fluids) bind to antigens (proteins on the cell surface of infectious agents such as viruses or bacteria) and thereby identify them for destruction or removal. There are 5 antibody or Ig classes in mammals: IgM, IgG, IgA, IgD, and IgE, each with a different structure and function. IgM is important for the primary response after the first exposure to an antigen and IgG is a latter response that is important in recognizing the antigen following re-exposure (National Toxicology Program, 2016).

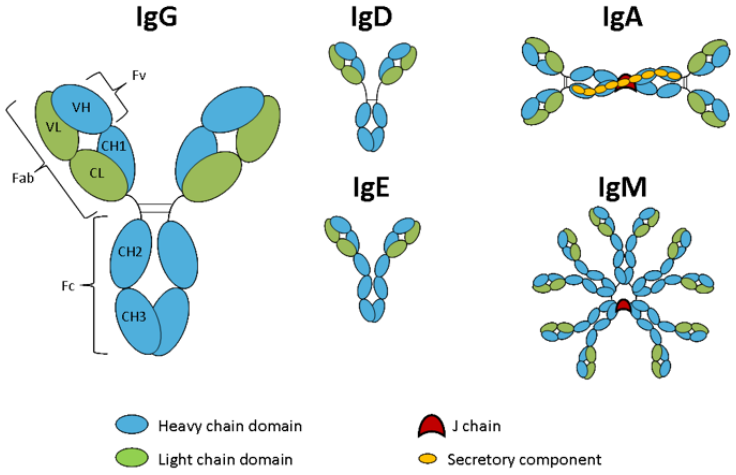


Figure 1.7 – Immunoglobulin isotypes
[Modified by: https://absoluteantibody.com]

Evidence provided by the available key studies show that higher developmental, childhood, or adult serum concentrations of PFOS are associated with lower specific antibody response to one or more commonly used vaccine in each study with reduced antibody response to diphtheria being the most consistent in all of the stages of life (Grandjean et al., 2012; Mogensen et al., 2015; National Toxicology Program, 2016; Kielsen et al., 2016).

A study in Faroe Islands confirmed negative associations between increased exposure to PFOS (geometric mean of 16, 7 ng/mL) through marine diet in 5-year-old children and antibody concentrations against both tetanus and diphtheria in the serum of the same children two years later (Grandjean et al., 2013). In the same study, maternal PFOS serum concentrations during the last trimester of pregnancy were found to be associated with anti-

diphtheria antibodies in 5-year-old children (geometric mean of 27,3 ng/mL) (Grandjean et al., 2013). Additional data on exposure at 7-years of age reported a greater reduction on the antibody levels after combining three major PFAAs (Perfluorooctanoic acid (PFOA), PFOS and Perfluorohexanesulfonic acid (PFHxS)) rather than the individual PFASs (Mogensen et al., 2015). A larger analysis among 12-19 year-old children observed decreases in anti-mumps and anti-rubella antibodies associated with increases in serum concentrations of PFOS (geometric mean of 4,13 ng/mL and 2,47 ng/mL) (Stein CR et al., 2015). Negative associations between serum levels of PFOS and anti-diphtheria antibodies were also reported among adults (Kielsen K. et al., 2016).

Exposure to PFOS associated with lower ability to resist or respond to infectious disease may provide additional insight on health outcomes potentially associated with reduced antibody response. However, only two human studies measured antibody response to vaccination and infectious disease outcomes in the same populations, but neither provided evidence for PFOS-associated increases in infectious disease (Granum et al., 2013; Looker et al., 2014). Despite that, Granum and colleagues studies reported maternal PFOS being associated with decreased antibody levels to rubella vaccination. In addition, no association between PFOS and episodes of common cold and gastroenteritis in children up to age three was observed (Granum et al., 2013). Recently though, the association between maternal serum concentrations of PFOS during pregnancy and the child's rate of hospitalization due to common infectious diseases between birth and 4 years of age was investigated (Dalsager L et al., 2021). Across types of infectious diseases, PFOS was associated with increased hospitalization rates. Their findings suggest that decreased resistance against infectious diseases occurs at levels of PFAS exposure similar to those that are associated with deficient vaccine responses (Dalsager L et al., 2021).

Studies based on data on children of age 9-12 suggested a general association of PFOS with decreased respiratory hypersensitivity (Humblet et al., 2014; Stein et al., 2016) while another one report observed an association between PFOS and increased self-reported food allergies (Buser and Scinicariello, 2016). Serum PFOS concentration was associated with lower allergen specific IgE levels (but no total serum IgE) for several allergens i.e. plants and cockroaches/shrimp, but increased specific IgE levels to mold suggesting a different impact of PFOS depending on the antigen (Stein et al., 2016; National Toxicology Program, 2016). On the contrary, PFOS was positively associated with total serum IgE, absolute eosinophil count and eosinophilic cationic protein concentration in a study among asthmatics in Taiwan (Dong et al., 2013; National Toxicology Program, 2016).

Furthermore, prenatal concentrations of PFOS (geometric mean of 3, 1 ng/mL) were negatively associated with an antibody directed against a self-protein in a small study in Faroese children (Osuna CE et al., 2014).

Animals

The majority of animal studies focus the attention on the measure of T-cell dependent antibody response (TDAR) after exposure to a xenobiotic. To do this, the latter are injected with an antigen and some days later, their blood is collected and primary antibodies (IgM) specific to the antigen, or secondary (IgG) antibodies after modification of the assay, are measured (Ladics GS, 2018).

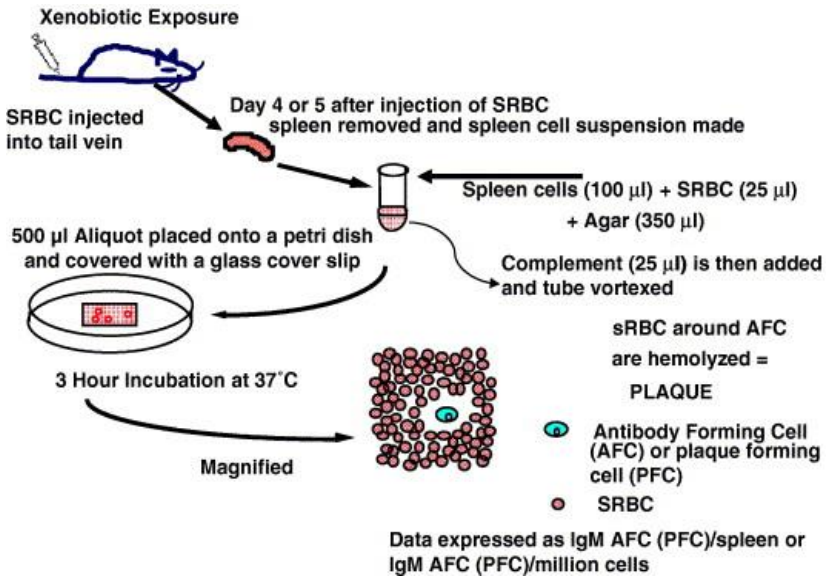


Figure 1.8 – T cell dependent antibody response (TDAR)
[Modified by: Ladics GS, 2018]

Generally reported, oral PFOS exposure leads to suppression of the antigen-specific IgM antibody production caused by T-cell specific antigens (SRBC) in male and female mice (Peden-Adams et al., 2008; Dong et al., 2009b; Zheng et al., 2009; Dong et al., 2011; Vetvicka and Vetvickova, 2013); along with a study in chickens supporting this evidence (Peden-Adams et al., 2009). Responses were less consistent depending on the oral doses moving from a wide range to smaller doses (0,002 to 40 mg/kg/day).

However, one study on male mice did not observe any change in the TDAR after exposure to 7mg PFOS/kg/day through a 28-day diet (Qazi et al., 2010b). Another study in male and female rats reported dose-responsive increase of the antigen-specific IgG antibodies only for male animals (Lefebvre DE et al., 2008).

The release of cytokines (like interleukin-4 (IL-4), IL-5, and IL-6), which are important for T-cell dependent antibody response was also examined in mice. The results reported that PFOS did not affect IL-4, IL-5, or IL-6 secretion by T-cells in mice, but increased IL-6 secretion from B-cells following anti-CD40 stimulation (Fair et al., 2011). PFOS exposure was associated with increased secretion of IL-4 and IL-6 in splenocytes from mice exposed to high PFOS doses associated with decreased antibody response (0.833 to 20 mg/kg/day) (Dong et al., 2011, Mollenhauer et al., 2011; Zheng et al., 2011; National Toxicology Program, 2016). Overall, PFOS affects cytokine secretion in various ways depending on the dose, cell type, and stimulation conditions.

To summarize, the human and animal studies provide a consistent pattern of findings that higher prenatal, childhood, or adult serum concentrations of PFOS are associated with suppression of the antibody response.

Multiple components of the immune system are related to disease resistance, and successful immune response to viral challenge includes rapid responses from the innate immune system (e.g., NK cell activity and the cytokine IFN-γ) as well as humoral immunity (antibody mediated responses) (National Toxicology Program, 2016). Dose-response with increasing PFOS exposure across multiple measures of disease resistance, such as lower body weight and increased mortality, was observed when mice were exposed to PFOS (0.005-0.05 mg/kg/day via gavage) for 21 days, indicating reduced resistance to influenza A virus challenge (Guruge et al., 2009). PFOS-associated reductions in antibody response in mice at similar exposure levels (0.002 to 40 mg/kg/day) and NK cell activity in mice at higher exposure levels (0.833 to 40 mg/kg/day) support the validity of PFOS associated suppression of disease resistance (Dong et al., 2011; Zheng et al., 2011; Dong et al., 2012). NK cells are important for resistance against viruses and tumor cells and they are good predictors for overall immunotoxicity.

In an *in vivo* study of PFOS immunotoxicity in mice (Lv et al., 2015), exposure to PFOS (10 mg/kg/day) for 4 weeks was found to inhibit the mitogenic reaction and thereby reduce the proliferation of T cells, which was explained by the downregulation in the gene expression of the cell cycle that was also observed in their experiments. After further analysis of several different pathways related to the signaling transduction of immune cells, PFOS-mediated inhibition of the NRF2-mediated pathways was observed (Lv et al., 2015). These pathways contribute to cell protection from oxidative damage, and upregulation of the gene expression in T cell receptor signaling, calcium signaling, and p38/MAPK signaling pathways. These signaling pathways play important roles in immune regulation. The

interference of these signaling pathways was considered a potential mechanism of PFOS-induced immunotoxicity (Lv et al., 2015; Zeng Z et al., 2019).

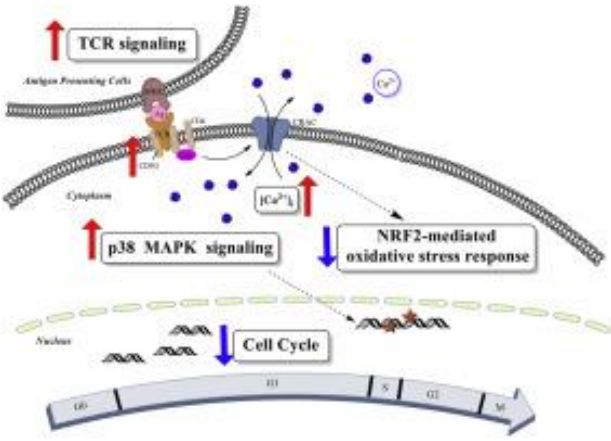


Figure 1.9 – The effect of PFOS on different pathways
[Modified by: Lv et al., 2015]

Concerning hypersensitivity effects, Dong and colleagues (2011) reported that oral PFOS exposure (0.8333 mg/kg/day) for 60 days was associated with increased antigen specific IgE levels following SRBC challenge. Dietary exposure to PFOS (4 mg/kg through 12 weeks of age) was associated with greater airway sensitivity to methacholine (drug used to diagnose bronchial airway hyper reactivity or asthma), but the association was not supported by other airway measures (e.g., no effect on airway resistance, tissue resistance, or elastance), while some results suggested suppression (Ryu et al., 2014; National Toxicology Program, 2016). Therefore, evidence of hypersensitivity reactions in experimental animals is considered inconsistent.

1.2.2. *In vitro* Methods

The advances in the fields of toxicogenomics, bioinformatics, epigenetics and computational toxicology could divert immunotoxicity testing from *in vivo* to methods based mainly on *in vitro* assays, using cells or cellular components, preferably of human origin (National Research Council, 2007). In this case, *in vivo* studies are useful to ascertain the toxic effects of PFOS on humans, while *in vitro* studies are important and effective means to understand its toxicity mechanism.

More in detail, and regarding the impact of PFOS on cytokine release, as described by Corsini and colleagues (2011, 2012), PFOS exposure (from 100 to 10000 ng/ml) resulted in lower IL-6 and IL-4 release in cultures of whole blood. As was shown prior (Corsini et al., 2011), the inhibitory effect of PFOS on *in vitro* cytokine production by human leukocytes occurs independently of the nuclear peroxisome proliferator-activated receptor PPARα despite the

fact that PFOS is known to activate this receptor. In further tests of potential mechanisms for the observed cytokine changes, Corsini et al. (2011, 2012) demonstrated that PFOS inhibited pathways that regulate NF-κB activation, which plays a role in cytokine production as well as apoptosis, inflammation, and other immune functions. In these studies, PFOS exposure at concentrations relevant to human exposure (100-10000 ng/ml PFOS, including the lowest dose tested) was also shown to reduce secretion of INF-γ (after phytohaemagglutinin stimulation)(Corsini et al., 2011; Corsini et al., 2012). Studies reporting PFOS-associated decreases of INF-γ from mouse splenocytes and human peripheral blood support the biological plausibility of effects on NK cell activity (Dong et al., 2011; Zheng et al., 2011; Dong et al., 2012).

Other studies reported that the inhibition of IL-2 production in human T cells was observed after exposure to 50, 75, and 100 mg/L of PFOS for 18 h in an in vitro study of PFOS-induced immunotoxicity (Midgett et al., 2015). IL-2 is a type of cytokine signaling molecule that regulates the immune activity of leukocytes, and the reduction of IL-2 is a characteristic of autoimmune diseases.

Potential mechanisms for PFOS-associated suppression of NK cell activity include reduced IL-2 mediated NK cell activation, reduced production of INF-γ by NK cells, and a role for NF-κB. No *in vitro* studies were found that tested the potential effects of PFOS on antibodies or antibody production (National Toxicology Program, 2016).

The study by Yamaki and Yoshino (2010) was the only one regarding the *in vitro* study of PFOS exposure to hypersensitivity-related endpoints. The authors suggest that the *in vitro* exposure to PFOS (5000 - 150000 ng/ml PFOS) led to increased release of hypersensitivity mediators from rat basophils (Yamaki and Yoshino, 2010). These results are similar to that reported with PFOA treatment, but cannot support any human and animal studies on PFOS-associated hypersensitivity as the latter are inconsistent (National Toxicology Program, 2016).

In conclusion, the *in vivo* and *in vitro* studies show that PFOS can interfere with the normal function of the immune cells and with the release and activity of immune active substances. However, information on modes of action and adverse outcome pathways must be expanded. It is significant to detect the structural and functional changes of the human body in the levels of molecules or cells, which could also provide insight on possible biomarkers for PFOS-induced toxicity/injuries.

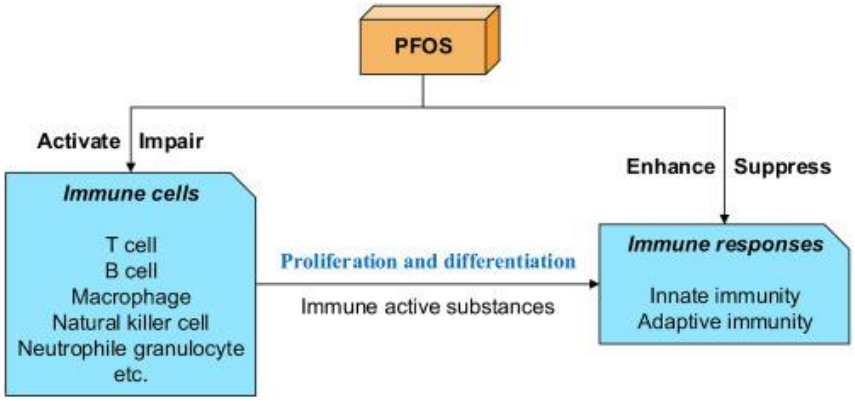


Figure 1.10 – Effects of PFOS exposure on the immune cells and immune responses.
[Modified by: Zeng Z et al., 2019]

2. SCOPE OF THE THESIS

Epidemiology and laboratory studies have shown that PFAS, such as PFOS and PFOA are able to induce alterations on several immune functions, suggesting their role as immunotoxic compounds. During the EFSA Scientific Opinion *"Risk to human health related to the presence of perfluoroalkyl substances in food"*, published in 2020 by the CONTAM Panel (EFSA, 2020), some gaps in PFAS immunotoxicity were identified. Specifically, data gaps are (I) the lack of information regarding the mechanisms underlying the observed immunosuppression effects (i.e., reduction in the vaccination efficacy and possible increase in the susceptibility to infectious disease) and (II) the assessment of a possible common mode of action and potency differences. There has been some progress regarding the assessment of toxic effects related to PFOS. However, more toxicological tests and data are needed to improve the knowledge about the long-term effects and mechanisms of PFOS toxicity. Moreover, PFOS can be used as reference PFAS, since it is one of the most widely used and studied chemicals in the PFAS group along with PFOA, in order to provide insight into the relative potencies of relevant PFAS. Based on the *in vivo* evidence of PFOA/PFOS-induced immunosuppression, suitable *in vitro* models that allow the evaluation of parameters relevant for the toxicity *in vivo* were identified, with particular attention to models reflecting antibody production.

The aim of this work is the *in vitro* evaluation of the immunotoxic effects of PFOS specifically on the maturation of DCs through the evaluation of possible effects on surface marker regulation.

3. MATERIALS AND METHODS

3.1. Experimental Model

3.1.1. CD40, CD80, CD83, CD86 and HLA-DR Expression as Markers Linked to the Maturation of DCs

DCs are important regulators of adaptive immunity. However, their use in in vitro methods has been difficult due to the low levels in the source and the high donor-to-donor variability. Nevertheless, it is possible to use monocytic derived cell lines; the most common one is the THP-1 cell line (Berges C et al., 2005).

Regarding the monocyte - derived DCs, CD80 and CD86 constitute powerful members of the co-stimulatory family. It has been shown that a variety of inflammatory or pathogen-derived mediators quickly up-regulate the expression of CD80 and CD86, therefore both molecules serve as very early co-stimulatory signals. In particular, the expression of co-stimulatory molecules (CD40, CD86 and CD80) at the DCs cell surface correlates with their ability to induce or suppress immune responses. In addition, CD40 is considered to up-regulate the expression of CD80 and CD86 (Manzoor A Mir, 2013; Hubo M et al., 2013).

As a result of maturation, DCs undergo a change in their morphology and develop cellular extensions that enlarge cellular surface and improve the interaction with T cells. However, the major events in DCs maturation are probably the up-regulation of MHC II and co-stimulatory molecules on their surface. Human CD83, together with CD86 and HLA class I and class II molecules, represent an important marker for the maturation of DCs.

CD80 and CD86

CD80 (also called B7-1) and CD86 (also called B7-2) are members of the Ig superfamily and are transmembrane glycoproteins. They consist of two extracellular Ig-like domains linked to a transmembrane domain and a cytoplasmic tail. The cytoplasmic tail contains three potential sites for protein kinase C phosphorylation, indicating a potential signaling role for this molecule(Lenschow et al., 1996).The role of PKC-β in allergen-induced CD86 expression in primary human monocyte-derived DCs was demonstrated in Corsini et al. (2014),indicating a central role of PKC-β in the initiation of chemical allergen-induced DCs activation and contributing to the understanding of its mechanism.

The extracellular domain is the interacting region with CD28 and CTLA-4 receptors present on the membrane of T lymphocytes, a link that enhances T cell proliferation(Lenschow et al., 1996). CD80 (B7-1) and CD86 (B7-2) are typically found on the surface of professional antigen-presenting cells such as DCs. Both B7 molecules are ligands of T cell critical co-stimulatory molecule CD28 and of an inhibitory receptor CTLA-4 (CD152), which competes with CD28 for B7.The interaction between CD80/CD86 and CD28 leads to enhanced activation of T cells, while interaction of CD80/CD86 with CTLA-4 triggers the suppression of DCs and regulatory T cells, which can prevent an immune response to self-antigen (Manzoor A Mir, 2013; Hubo M et al., 2013).

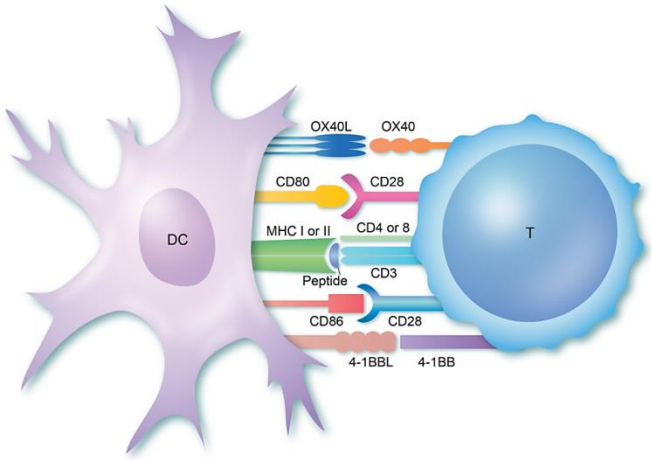


Figure 3.1 – Interaction between CD80/CD86 on DC and CD28 on T cells
[Modified by: https://www.immunology.org/public-information/bitesized-immunology/systems-and-processes/t-cell-activation]

CD40

CD40 is a transmembrane glycoprotein surface receptor and a member of the Tumor Necrosis Factor Receptor superfamily (TNFRSF). CD40 signaling induces changes in DCs, such as up-regulation of MHC class II and co-stimulatory molecules CD80/CD86, which make them more effective APCs. In addition, they promote the production of T cell stimulatory cytokines and engage in interactions with other cell types such as B cells leading to B cell class switching and secretion of IgG and IgA antibodies (Hubo M et al., 2013; Ma DY, 2009).

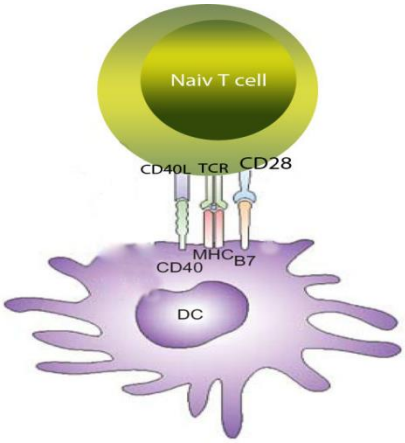


Figure 3.2 – Schematic presentation of CD40 and CD40L expression on DCs and Lymphocytes.
[Modified by: Karimi MH, 2012]

CD83

CD83 is glycoprotein member of the Ig superfamily predominantly expressed on numerous activated immune cells, including B and T lymphocytes, monocytes and DCs. It is composed of an extracellular V-type Ig-like domain, a transmembrane domain, and a cytoplasmic tail. It may serve an important role in antigen presentation or cellular interactions as suggested by the expression pattern of this molecule and its structural similarity with other members of the Ig superfamily. More specifically, its presence on the surface of activated APCs has been shown to cause an up-regulation of MHC class II and CD86 required for T cell activation. Despite that, signaling through surface CD83 leads to suppressed or regulatory functions in various immune cell populations (Zhou LJ and Tedder TF, 1995; Li Z, 2019).Most importantly though, CD83 is one of the most prominent surface markers for the maturation of DCs since CD83 cell surface expression levels are not detectable on immature DCs (Cao W et al., 2005; Grosche L et al., 2020).

HLA-DR

Human Leukocyte Antigen – DR isotype (HLA-DR) is a cell surface receptor belonging to MHC II and it is encoded by the human leukocyte antigen complex on chromosome 6p21 region. It is mainly found on APCs like DCs and macrophages, and these molecules play a key role in extracellular antigen presentation to CD4+ T cells, thus initiating the adaptive immune response. It has been also shown that there is a strong association between the HLA class II region and autoimmune disease including rheumatoid arthritis, type 1 diabetes and Graves' disease(Choo, 2007; Gough SC 2007). Furthermore, HLA-DR induced expression by extreme allergens has been investigated recently, suggesting their ability to induce a higher degree of

DC maturation. Moderate and weak skin sensitizers could induce the first signal of the T cell activation process (HLA-DR expression) only in the presence of keratinocytes (Galbiati et al., 2020). Moreover, the measurement of HLA-DR can provide an indication of a drug able to activate DCs when combined with the THP-1 activation assay, contributing to the classification of the drugs as sensitizers (Iulini et al., 2020).

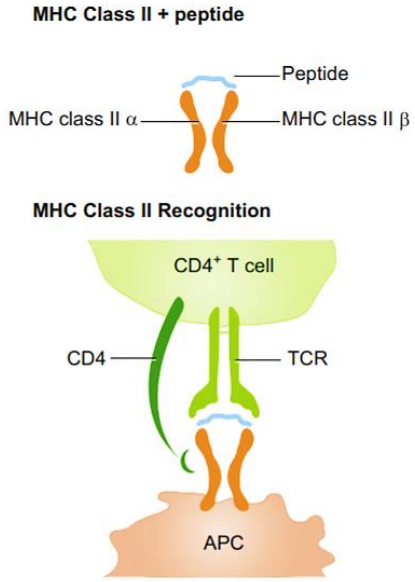


Figure 3.3 - The MHC II structure and interaction with TCR
[Modified by: Thompson, 2014]

3.2. Materials

3.2.1. Perfluorooctanesulfonic acid (PFOS)

3.2.1.1. Physicochemical Properties and Structure of PFOS

PFOS (CAS# 1763-23-1,~40% in H_2O Sigma – Aldrich, St. Louis, Missouri, USA) is soluble in dimethyl sulfoxide (DMSO) (CAS# 67-68-5,Sigma - Aldrich) that was used as a vehicle (final concentration in culture medium<0.1%) for the experiments. The concentrations tested were in the range of 1ng/ml to 10 µg/mL, with cell viability greater than 90% after 72 hours of treatment. It is perfluoroalkanesulfonic acids that is octane-1-sulfonic acid within which all seventeen of the hydrogens are attached to carbons have been replaced by fluorines (Rayne et al., 2008).

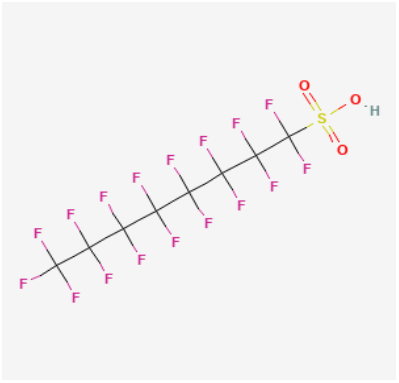


Figure 3.4 – Chemical structure of PFOS
[Modified by: https://pubchem.ncbi.nlm.nih.gov/compound/Perfluorooctanesulfonic-acid]

3.2.1.2 Metabolism and Pharmacokinetics

Per- and polyfluoroalkyl compounds, such as PFOS, have been shown to be readily absorbed in the gastrointestinal tract in mammals, including humans, to distribute mostly to the plasma and be retained in the liver (OECD, 2002). PFOS is not metabolized and is excreted in both urine and feces or undergoes extensive intestinal reabsorption (OECD, 2002). However, pharmacokinetic differences have been observed in laboratory animals. For example, the half-life is 100 days in rats, 200 days in monkeys, and years in humans. Differences in biological half-lives between species for PFOS are mainly due to differences in renal clearance (OECD, 2002; Knutsen et al., 2018; Schrenk et al., 2020).

3.2.2. Dexamethasone

3.2.2.1. Physicochemical Properties and Structure of Dexamethasone

Dexamethasone (DEX) (CAS# 50-02-2, Sigma-Aldrich) is a fluorinated steroid. It is a synthetic member of the class of glucocorticoids. It acts as an adrenergic agent, an antiemetic, an environmental contaminant, a xenobiotic, an immunosuppressive agent, and an anti-inflammatory drug (Elks J, 2014).

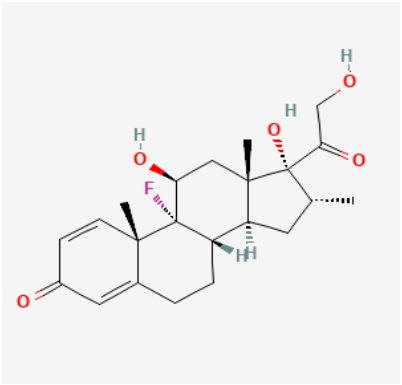


Figure 3.5 – Chemical structure of DEX
[Modified by: https://pubchem.ncbi.nlm.nih.gov/compound/Dexamethasone]

3.2.2.2 Pharmacology and Biochemistry

Corticosteroids bind to the glucocorticoid receptor, inhibiting pro-inflammatory signals, and promoting anti-inflammatory signals. Therefore, they are used as anti-inflammatory agents and as immunosuppressive agents. Corticosteroids can suppress the immune response in humans by inhibiting differentiation, terminal maturation and the function of DCs. More specifically, DEX is shown to affect culture-induced maturation by inhibiting the CD86 and CD83 increase and the secretion of some cytokines (Piemonti et al., 1999; Matasić et al., 1999). Therefore, in this experiment it was used as a positive control due to its immunosuppressive properties.

3.2.3 Cell line

The THP-1 cell line (Elabscience Biotechnology Inc.- Houston, Texas, USA) was isolated from the peripheral blood of a 1-year-old male acute monocytic leukemia patient (Auwerx, 1991). THP-1 can be converted into mDCs when cultured in medium containing IL-4, GM-CSF, TNF-a, and Ionomycin (Berges et al., 2005). Thus, it serves as an experimental model to study immune responses.

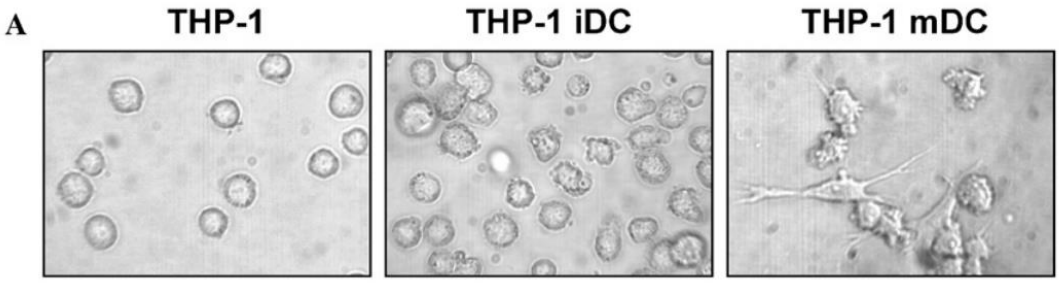


Figure 3.6 – Morphology of THP-1 cells (THP-1) and THP-1-derived immature (THP-1 iDC) and mature (THP-1 mDC) DCs
[Modified by: Berges et al., 2005]

3.3. Methods

3.3.1. Cell culture

To provide a highly reproducible model for human DCs differentiation, the THP-1 cell line was used.

THP-1 cells grow in suspension and are passaged every 3-4 days in Petri dishes (ø 100 mm, Corning® Costar®) with a density of 0.1 to 0.2 x 10^6 cells/mL and are maintained at a concentration between 0.1 to 1 x 10^6 cells/mL using RPMI-1640 culture medium with phenol red (Sigma-Aldrich) containing 2 mM L-glutamine (CAS# 56-85-9, Sigma-Aldrich), 0.1 mg/mL Streptomycin (Sigma-Aldrich) with 100 IU/mL Penicillin (Sigma-Aldrich), 0.1% of gentamicin (Sigma-Aldrich) and 0.1% of 2-mercaptoethanol(CAS# 60-24-2, Bio-Rad Laboratories, Hercules, California, United States) and supplemented with 10% of heat-inactivated Fetal Bovine Serum (FBS, Sigma-Aldrich). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

During the treatment, THP-1 cells are transferred from a Petri dish into a 50 mL propylene tube and then centrifuged at 1200 rpm for 5 minutes at T = 25 °C. The pellet obtained is then resuspended in 5 mL of new fresh culture medium. To perform the cell count, 10 µL of cell suspension were added to a solution containing 80 µL of sterilized Phosphate Buffered Saline (PBS)(Sigma-Aldrich) and 10 µL of Trypan Blue Solution 0.04% (CAS# 72-571, Sigma-Aldrich). Trypan Blue is a dye used as a cell stain. Live cells do not allow the dye to permeate the cell membrane, while cells taking up the dye and appearing blue, are considered non-viable. This way only the viable cells are counted, by excluding the colored cells. The count was performed manually by using a hemocytometer, and 10 µL were taken and placed in the Neubauer chamber. The cell count was carried out under an optical microscope by counting the living cells of the four quadrants and calculating their average number. The number of cells per mL (cc/mL) is calculated by applying the following formula:

$$\text{n°cc/mL} = \text{n°average cc} \times 10^4 \times 10$$

where 10^4: the factor of the Neubauer chamber and 10: the dilution factor.

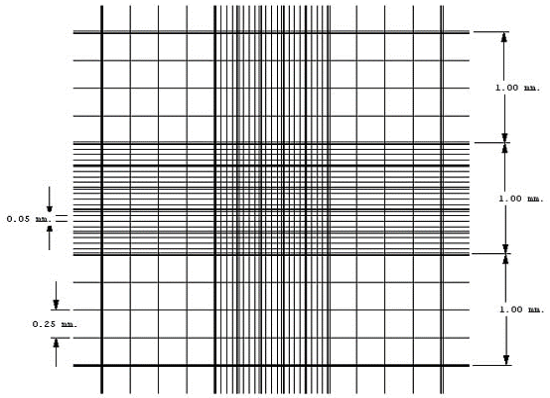


Figure 3.7 – The Neubauer chamber

[Modified by: https://www.emsdiasum.com/microscopy/technical/datasheet/63510.aspx]

To calculate the volume of cell suspension needed, the following equation was used: $C_1 \times V_1 = C_2 \times V_2$, where C_1 is the cell concentration obtained from the cell count (average of cells x 10^5 cells/mL), C_2 is the final desired concentration (i.e. 2 x 10^5 cells/mL), V_2 is the desired final volume of cells and V_1 is the volume of cell suspension needed. The volume of culture medium needed is calculated from the equation: $V_{medium} = V_2 - V_1$

3.3.2. Introduction In Flow Cytometry Analysis

Flow Cytometry is a technique to detect and measure in a population of cells physical and chemical characteristics. In the process, a sample containing cells in suspension is injected in the flow cytometer instrument and they subsequently flow in a fluid stream through a beam of light. The properties measured include size, granularity or internal complexity, such as the content of nucleic acids (DNA), the expression of intracellular proteins and surface receptors. Two main advantages of flow cytometry are that it is possible to simultaneously quantify multiple parameters cell by cell and that the measurements are performed accurately for about 10.000 cells in less than one minute.

The flow cytometer consists of three main elements:

1. A fluidic system that is responsible to transport cells from the sample to the flow chamber in a single file.
2. The optical system consists of various filters, lenses, detectors and the light source, which is usually a laser beam producing a single wavelength of light at a specific frequency. Through this laser beam the cells are passed one by one, which causes them to scatter or emit light, signals that are collected and filtered to be analyzed.
3. An electronic system that converts the detected light signals into electronic signals that can be processed by the computer. Thus, signals are digitized and saved in the computer for subsequent data analysis.

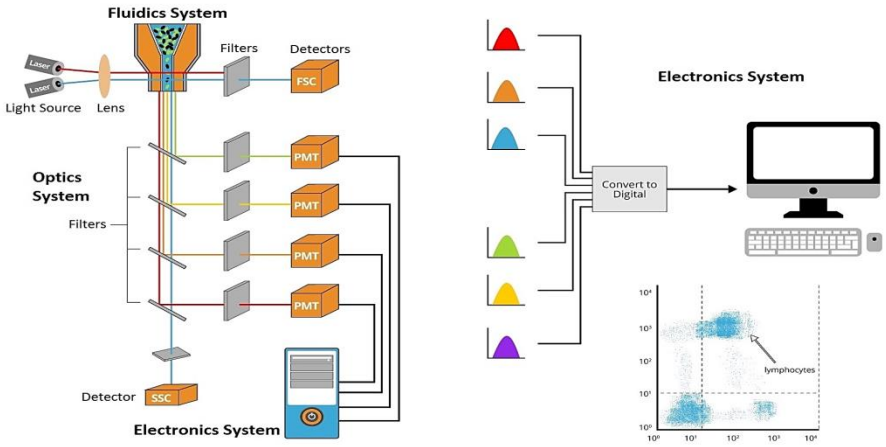


Figure 3.8 – Schematic representation of a Flow Cytometer.
[Modified by: https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle]

The main principle of this technique is based on scattering of light and emission of fluorescence, which occur after the passage of cells in a single file past a laser beam. For each cell, an independent event is recorded that is translated as a digital signal on a graph. There are two sensors that capture the scattered light:

- The forward scatter channel (FSC), which is proportional to the size of the cell.
- The side scatter channel (SSC), which relates to the complexity or granularity of the cell.

The data produced can be visually represented in different types of data plots. The most common types of data graphs used in flow cytometry include dot plots, histograms, and density plots. Correlated measurements of the two signals (FSC and SSC) allow obtaining a dispersion diagram (dot plot) in which different cell populations can be differentiated, based only on physical characteristics.

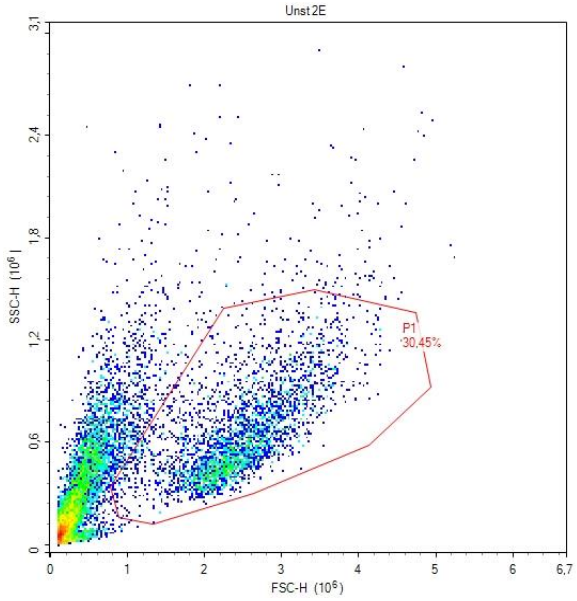


Figure 3.9 – A dot plot representation of iDCs

It is also possible to mark cells with fluorescent dyes, like a fluorophore, that will emit fluorescent light at specific wavelengths. A fluorescent dye conjugated to a monoclonal antibody can be used to identify a particular cell type based on the individual antigenic markers of the cell by absorbing light energy and releasing it in the form of a specific wavelength of light. Fluorophores absorb light at a certain wavelength and emit at wavelengths longer than the absorption one (Stoke's shift). Fluorochromes can be used simultaneously if the peak emission wavelengths are not extremely close to each other and if each one is excited at 488 nm (blue laser). The combination of FITC and phycoerythrin (PE), which satisfy these criteria, were used in this work:

- Fluorescein isothiocyanate (FITC) with an excitation peak at 495 nm and an emission peak at 519 nm.
- Phycoerythrin (PE) with an excitation peak at 566 nm and an emission peak at 576 nm.

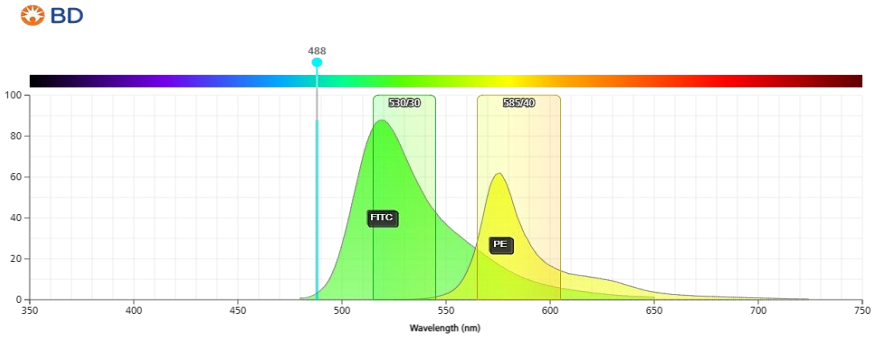


Figure 3.10 – Emission spectrum of the fluorophores FITC and PE.
[Modified by:https://www.bdbiosciences.com/en-it/resources/bd-spectrum-viewer]

During the analysis, a certain level of auto fluorescence by the cells is expected, which leads to false positive events. The level of auto fluorescence can be determined using unstained controls, useful to identify and gate the correctly stained cells during data analysis.
To determine the nonspecific binding of the fluorophore, isotype controls can be used. Isotype controls are antibodies raised against an antigen not found on the cell type analyzed and ensure that the observed staining is due to antibody binding to the target rather than an artifact.
The ideal isotype control should match:

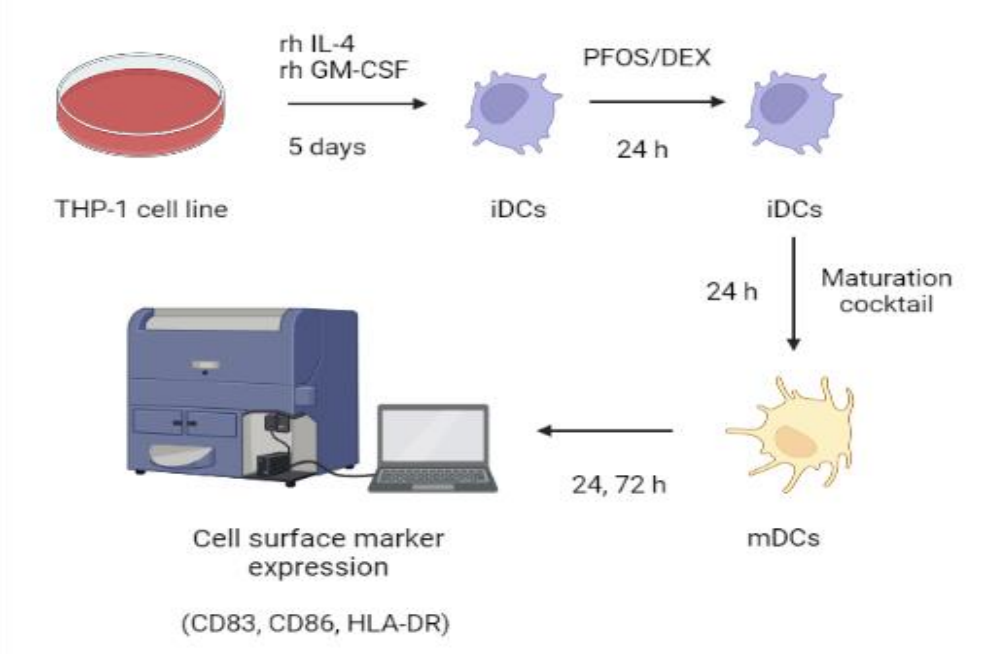
- The host species
- Ig subclass (IgA, IgG, IgD, IgE, or IgM) of the primary or secondary antibody
- Conjugated to the same fluorophore as the primary antibody (i.e. FITC, PE)

Moreover, single parameter histograms can be used to further identify distinct cell types that express a particular marker in a specific population of cells. Commonly, the Y-axis is the number of events that show a particular fluorescence and the X-axis is the fluorescence intensity detected in a single channel. Ideally, only one distinct peak will be produced that represents the large number of events detected at the specific intensity.
In addition, this type of plot can also be used to easily exclude dead cells by using a viability dye such as propidium iodide. Thus the dead cells would be positive for the dye.

3.3.3. Experimental workflow

The workflow followed can be summarized as:

1. Differentiation of THP-1 cells to immature dendritic cells.
2. Setting the best conditions for obtaining mature dendritic cells
3. Treatment of iDCs with PFOS one day before adding the maturation cocktail
4. Addition of the maturation cocktail to obtain mature dendritic cells
5. Flow cytometry analysis to check the surface marker expression



Created with BioRender.com

Differentiation from THP-1 to iDCs through Flow Cytometry

After the count of the cells, as previously described, the cells were brought at the concentration of 2 x 10^5 cc/mL. After those, two different flasks with the volume needed prepared for THP-1 and iDCs, respectively. To induce differentiation, in the flask namely iDCs, rhIL-4 (100 µg/mL) and rhGM-CSF (100 µg/mL) were added (Immunotools, Friesoythe. Germany). Cells were cultured for 5 days in order to acquire the properties of iDCs with two medium changes within these days.

After 5 days of differentiation, cells were transferred to flow cytometry tubes and centrifuged at 1200 rpm for 5 minutes at T = 25 °C. The supernatants were eliminated, while the cells left in the tubes were resuspended in 200 µL of PBS to which specific conjugated FITC antibodies were added (3 µL for the markers or 1.5 µL for the isotype):

- Mouse antihuman CD40 (CAS# 568580, BD-PharMingen™)
- Mouse antihuman CD80 (CAS# 555682, BD-PharMingen™)
- Mouse antihuman CD86 (CAS# 555663, BD-PharMingen™)
- Mouse IgG1 κ Isotype Control (CAS# 555748, BD-PharMingen™)

The tubes were stored for 30 minutes at 4 °C, protected from the light and subsequently centrifuged at 1200 rpm for 5 minutes at T = 25 °C. The supernatant was discarded, and the pellet was resuspended in 500 µL of PBS.

The mean fluorescence intensity (MFI) was analyzed using NovoCyte 3000 (Acea Biosciences, Inc.) on a sample of 10,000 cells and the data were quantified using the NovoExpress software. Changes in surface marker expression to evaluate the differentiation from THP-1 to iDCs are expressed as stimulation index (SI) calculated on the mean values (treated cells/vehicles treated cells).

$$SI = \frac{Mean\ Fluoresence\ positive\ treated\ cells - Mean\ Fluoresence\ Isotype\ treated\ cells}{Average\ (Mean\ Fluoresence\ \ positive\ control\ cells - Mean\ Fluoresence\ \ Isotype\ control\ cells)}$$

Treatment with the Chemical

Preparation of the chemical stock (10mg/ml)

PFOS is liquid with a concentration of 40% in H_2O and a molecular weight of 500,13.

Considering an amount of PFOS around 30µL and weighing it, 36,2 mg of PFOS is obtained.

Dividing that amount of the chemical with the volume of 30µL to get a concentration of 1206,66 mg/mL and then multiplying by 40%, 482,67 mg/mL is calculated to be the concentration of the chemical. By using the formula $M_1xV_1=M_2xV_2$, where M_2: 10mg/mL, V_2: 1mL, the stock was prepared by adding 20,72 µL of the chemical in 979,28 µL of DMSO.

In order to obtain the desirable concentrations of PFOS, the different working solutions that were the 1000X of the concentrations needed inside the cells were prepared starting from the stock (10mg/ml).

In the treatment DEX was also used as positive control. The amount of DEX was calculated by using the formula: $C_1 \times V_1 = C_2 \times V_2$,

with C_1: 75 mg/mL, C_2: 150 µg/mL and V_2: volume of the iDCs in the well.

Cell Treatment

After 5 days of incubation as mentioned above, THP-1 cells and iDCs were transferred from the corresponding flasks, under the lateral flow hood, into propylene tubes, and then centrifuged at 1200 rpm for 5 minutes at T=25 °C.

The pellet was resuspended in 5 ml of RPMI-1640 medium without phenol red (Sigma-Aldrich) containing 2 mM L-glutamine, 0.1 mg/mL Streptomycin, 100 IU/mL 0.1% of gentamicin and 0.1% of 2-mercaptoethanol, and two cell counts were performed separately for THP-1 and iDCs as described previously. Then the cells were brought to the final concentration of $1x10^6$ cc/mL and plated in a 12-well plate and treated with chemicals and vehicle control. The conditions used are:

- THP-1

- iDCs
- iDCs with DMSO
- iDCs with DEX (150μg/ml), used as positive control
- iDCs with the different PFOS concentrations (1ng/ml – 10μg/ml)

Maturation of DCs

24 hours after the treatment with chemicals alone, mDCs were generated from iDCs by addition of maturation cocktail composed by:

- rh-IL-4: 1 μg/ml
- rh-GM-CSF: 500 ng/ ml
- Tumor necrosis factor (TNF)-α: 100 ng/ml
- Ionomycin: 1μg/ml

Analysis of mDCS through Flow Cytometry

24 hours and 72 hours after adding the maturation cocktail, cell-surface expression of CD83, CD86 and HLA-DR was determined by flow cytometry analysis.
Cells were transferred to flow cytometry tubes and centrifuged at 1200 rpm for 5 minutes at T=25°C.The supernatants were eliminated, while the cells left in the tubes were resuspended in 200 μL of PBS to which specific conjugated FITC or PE antibodies were added(3 μL for the markers or 1μL for the isotype) (BD-PharMingen™):

- Mouse antihuman CD86 conjugated with FITC
- Mouse antihuman CD83 conjugated with PE
- Mouse antihuman HLA-DR conjugated with FITC
- Mouse IgG1 κ Isotype Control conjugated with FITC
- Mouse IgG1 κ Isotype Control conjugated with PE

Same as before, the tubes were stored for 30 minutes at 4 °C, protected from the light and subsequently centrifuged at 1200 rpm for 5 minutes at T = 25 °C. The supernatant was discarded, and the pellet was resuspended in 500 μL of PBS.
The MFI was analyzed using NovoCyte 3000 on a sample of 10,000 cells and the data were quantified using the NovoExpress software. Changes in surface marker expression are expressed as stimulation index (SI) calculated on the median values as previously described.

3.3.4. Data show

The data presented are expressed as mean ± standard error of the mean (SEM) with *n = 2* independent experiments.

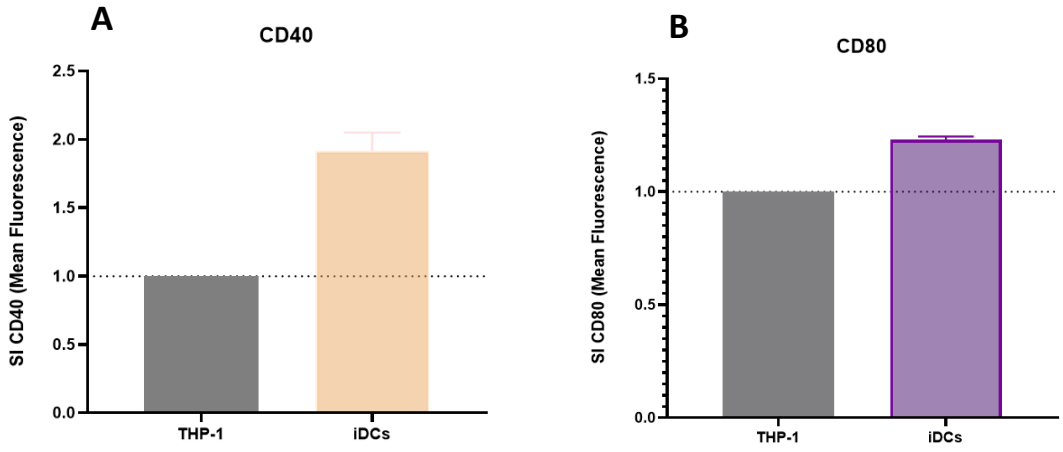
4. RESULTS

4.1 Differentiation of THP-1 cells to iDCs

The first aspect of this work was to check the differentiation of THP-1 cells to iDCs. To evaluate the expression of the surface markers CD40, CD80 and CD86, the data obtained by flow cytometry were expressed as SI in reference to the positivity of this marker on treated cells, using the following formula:

$$SI = \frac{Mean\ Fluoresence\ positive\ treated\ cells - Mean\ Fluoresence\ Isotype\ treated\ cells}{Average\ (Mean\ Fluoresence\ \ positive\ control\ cells - Mean\ Fluoresence\ \ Isotype\ control\ cells)}$$

The expression levels of CD40, CD80 and CD86 are increased in comparison to the control of the differentiation (THP-1 with SI=1). Therefore, it can be stated that the differentiation of the THP-1 cells to iDCs has been achieved.



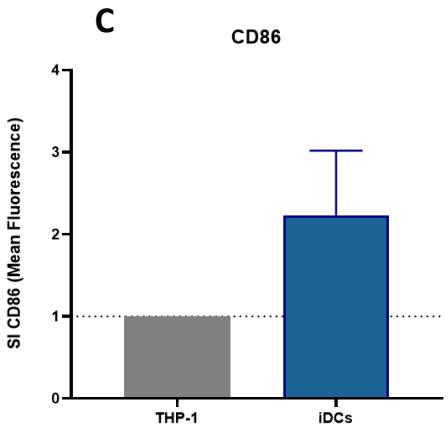
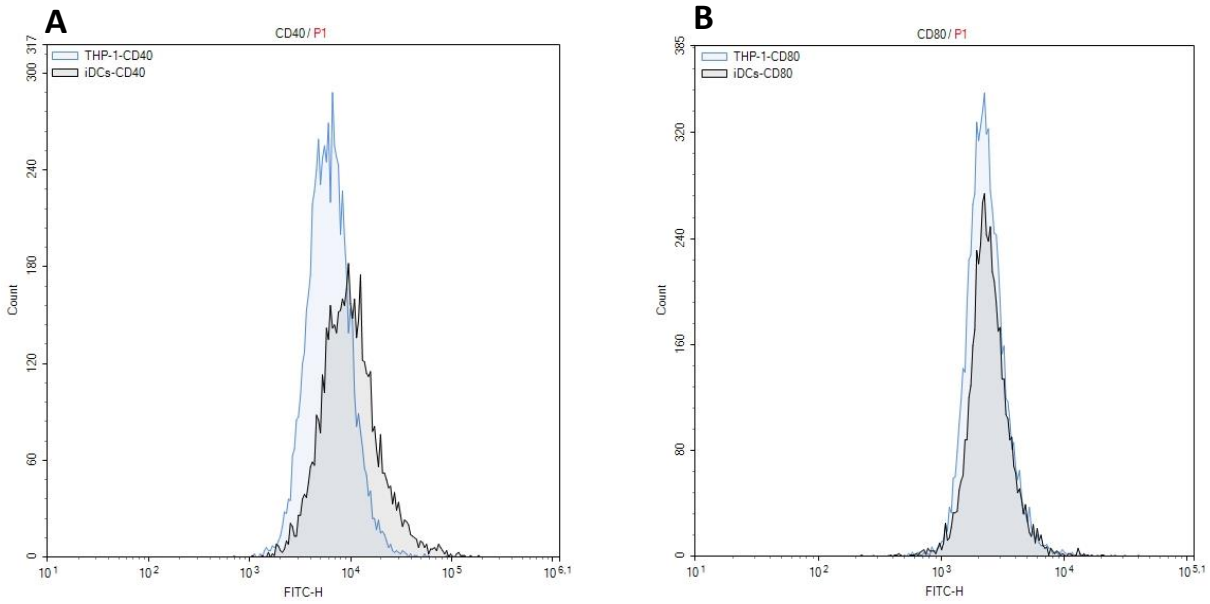


Figure 4.1 - The Expression of the surface markers CD40 (Fig.4.1 A), CD80 (Fig. 4.1 B) and CD86 (Fig. 4.1 C) on iDCs compared to THP-1 cells.

THP-1 cells were cultured for 5 days. To induce differentiation, rh-IL-4 and rh-GM-CSF were added in the flask. The results are expressed as stimulation index (SI). The dotted line shown on the y-axis corresponds to the differentiation control (THP-1). The expression levels of the three surface markers are increased in comparison to the control of the differentiation (THP-1 with SI=1), confirming the differentiation of THP-1 cells into iDCs. Each value shown represents the mean ± standard error of the mean (SEM), with *n = 2* independent experiments.



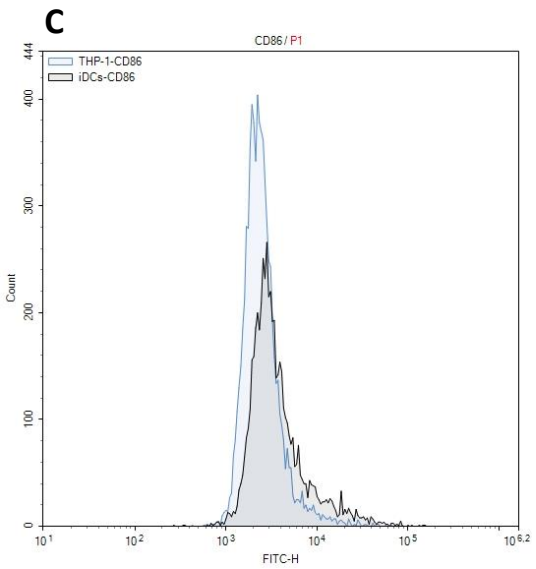


Figure 4.2 – Histograms profiles of iDCs

FACs analysis shown in histograms was performed to determine the expression of the cell surface markers CD40 (Fig. 4.2 A), CD80 (Fig. 4.2 B) and CD86 (Fig. 4.2 C) during the generation of the immature DCs starting from THP-1 cells in the presence of rh-IL-4 and rh-GM-CSF. The differentiation is confirmed observing the overlay of iDCs and theTHP-1 cells.

4.2 Maturation test

An issue that should be noted is that the absence of serum and phenol red does not successfully lead to the maturation of DCs after adding the maturation cocktail, as confirmed by the analysis of the cells through flow cytometry. Moreover, aggregation of the cells was observed, after the PFOS treatment. Consequently, due to the different morphology of those cells, it was not possible to detect them within the gate of the dot plot as shown in Figure 4.3B.

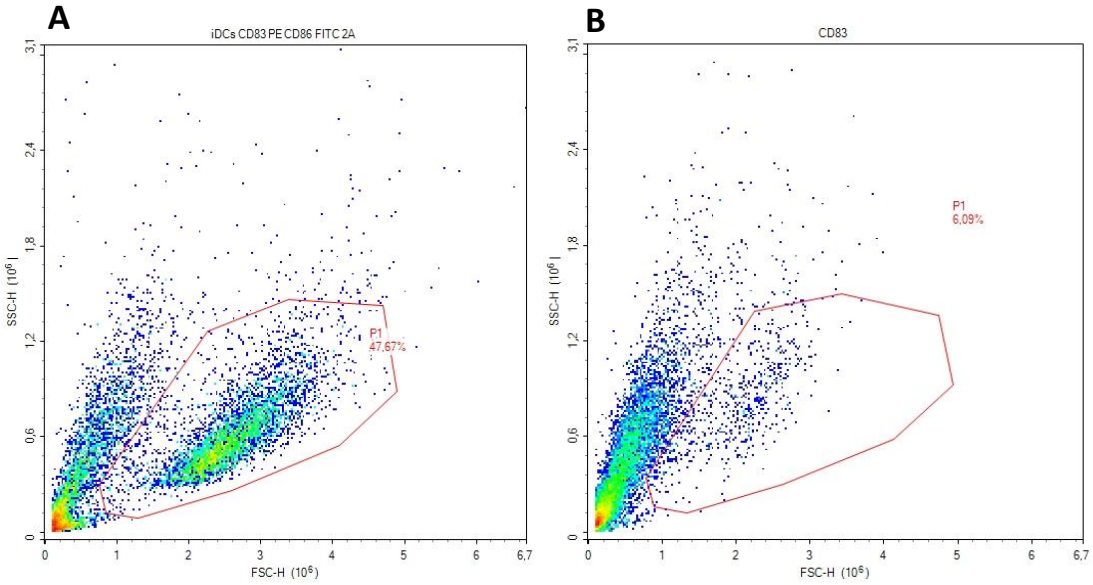


Figure 4.3 - Dot plot of iDCs before (figure 4.3 A) and after (figure 4.3 B) addition of the maturation cocktail in serum-free medium.

Dot plot showing FACs analysis of DCs (labeled with CD83 PE) after the addition of the maturation cocktail in serum-free medium. As observed in Figure 4.3 A, the percentage of iDCs is around 50%, but decreases down to 6% after the addition of the maturation cocktail, as shown in Figure 4.3 B, making the analysis of the cells impossible.

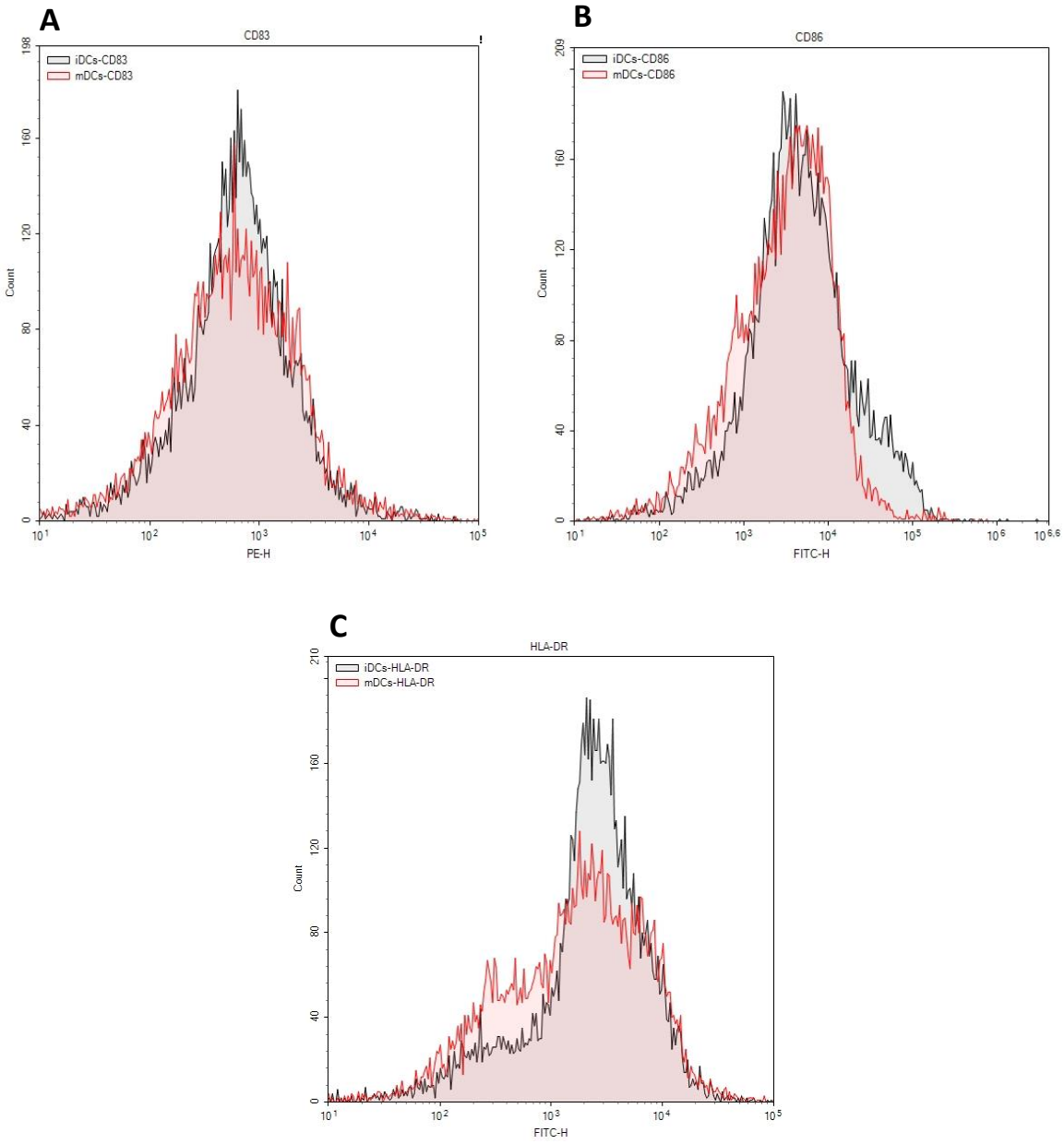


Figure 4.4–Histogram profiles of iDCs and mDCs in serum-free medium

FACS analysis, shown in histograms, was performed to determine the expression of the cell surface markers CD83 PE (Fig. 4.4 A), CD86 FITC (Fig. 4.4 B) and HLA-DR FITC (Fig. 4.4 C) on mDCs after the addition of the maturation cocktail in serum-free medium.

As shown in the overlay histograms of the latter compared to the control iDCs, the maturation was not obtained under these conditions.

Therefore, requirements for the maturation of DCs in culture had to be determined. The aim was to obtain terminally differentiated, mDCs. For this purpose, one independent

experiment was performed as a test in order to define the best conditions for the maturation of the DCs.

The workflow that was followed for this experiment includes the same steps as were mentioned above until before the PFOS treatment.

The conditions tested, were the following:

- concentration of cells : 1 x 10^6 resuspended in RPMI medium without phenol red and without serum
- concentration of cells : 1 x 10^6 resuspended in RPMI medium without phenol red and with 5% of delipidated FBS
- concentration of cells : 2 x 10^5 resuspended in RPMI medium without phenol red and without serum
- concentration of cells : 2 x 10^5 resuspended in RPMI medium without phenol red and with 5% of delipidated FBS

The maturation cocktail was added based on the two different concentrations of the cells and the cells were incubated for 24 hours and 72 hours. Flow cytometry analysis was used to determine the best conditions in order to obtain mDCs.

By comparing the data obtained by this test, it was decided that the best concentration of the cells for the following experiments would be 1 x 10^6 resuspended in RPMI medium without phenol red and with 5% of delipidated FBS, since under these conditions the maturation was definitely obtained both after 24 and 72 hours of treatment.

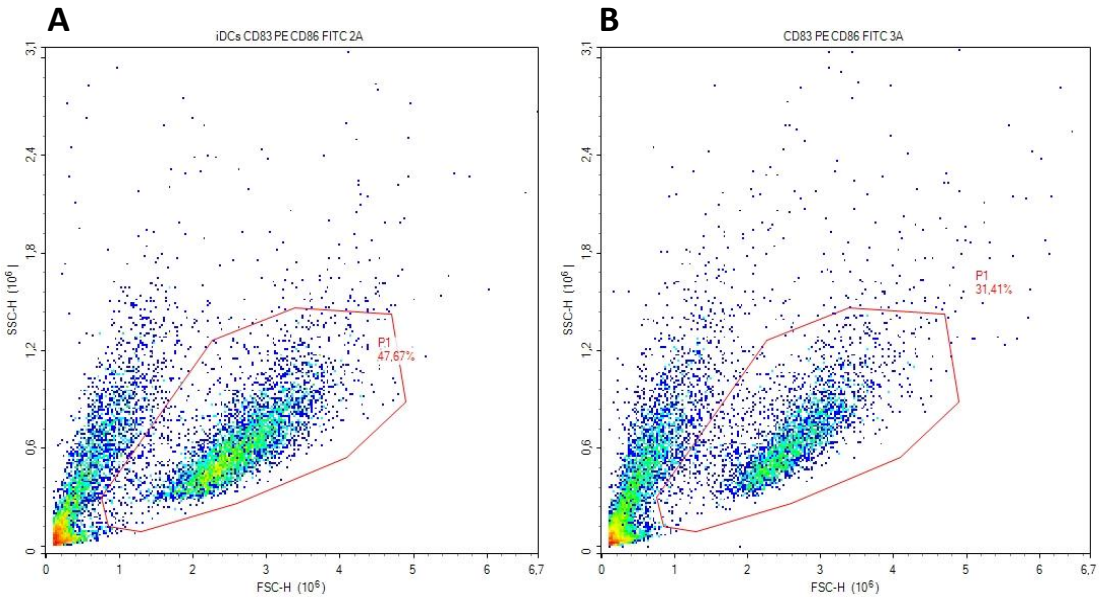


Figure 4.5 - Dot plot of iDCs (figure 4.5 A) and mDCs (figure 4.5 B).

Dot plot showing FACs analysis of DCs (labeled with CD83 PE and CD86 FITC) after the addition of the maturation cocktail in serum-containing medium. There is a decrease from around 50% (Fig. 4.5 A) to 30% (Fig. 4.5 B). The decrease on the percentage of cells is lower than the initial conditions confirming that the working conditions are good.

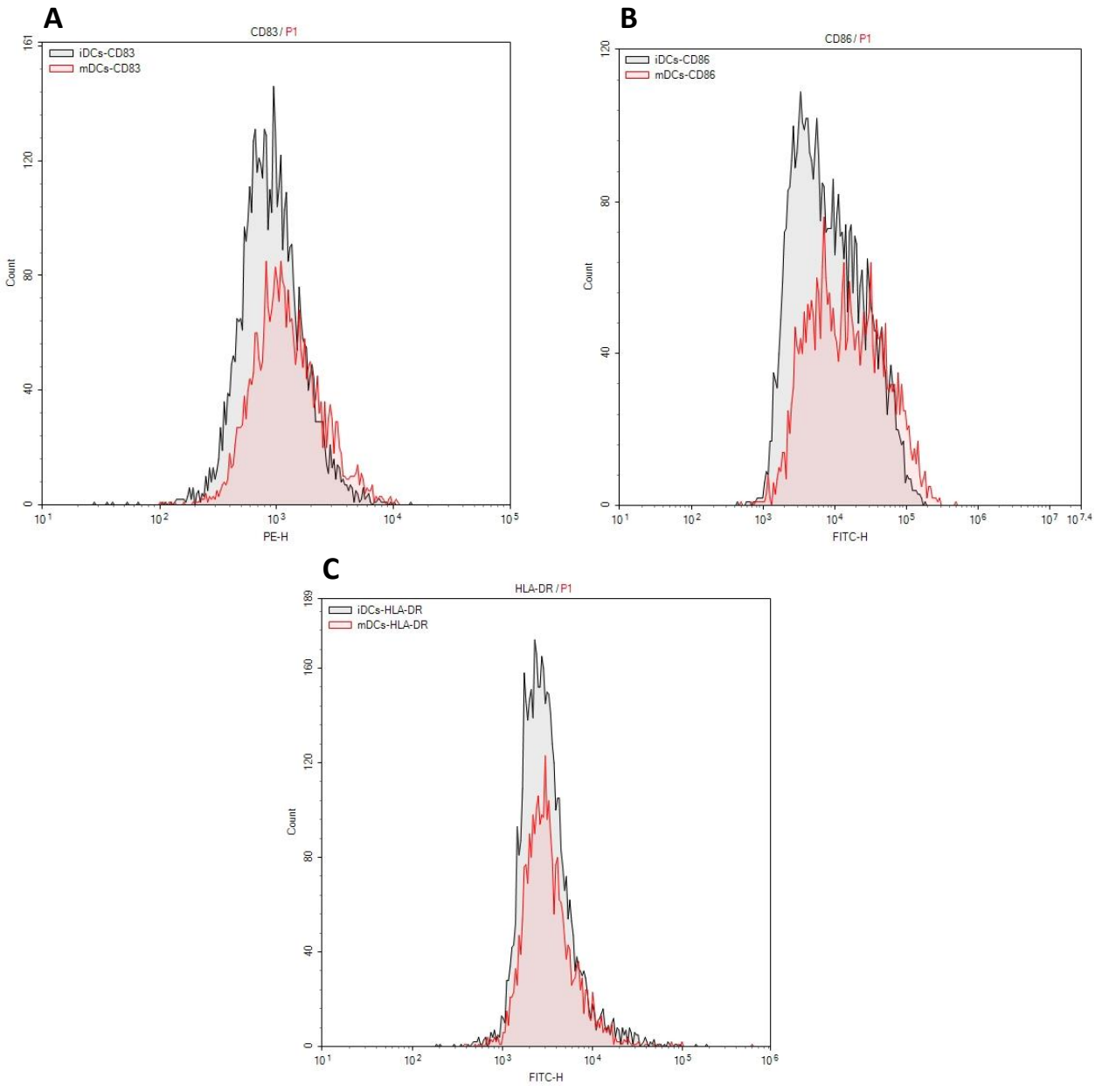


Figure 4.6 – Histogram profiles of iDCs and mDCs in medium plus 5% of delipidized FBS.

FACs analysis shown in histograms was performed to determine the expression of the cell surface markers CD83 (Fig. 4.6 A), CD86 (Fig. 4.6 B) and HLA-DR (Fig. 4.6 C) on mDCs. The overlay histograms of mDCs compared to the control iDCs confirm that the maturation

was obtained under the new conditions decided after the maturation test (concentration of cells: 1 x 10^6 resuspended in serum-containing medium without phenol red).

4.3 Effects on the Expression of CD83, CD86 and HLA-DR

In order to evaluate the immunotoxic potential of the tested chemical on the maturation of the DCs, the change in the expression of CD83, CD86 and HLA-DR was analyzed by flow cytometry.

iDCs were treated with PFOS concentrations of 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/ mL and 10 µg/mL. 24 and 72 hours after the addition of the maturation cocktail, the cells were analyzed by flow cytometry. The concentrations tested were assessed first through a CV75 viability test (results not shown).

In all the graphs, the expression of all the markers is induced for the untreated mDCs meaning that the maturation of DCs was obtained.

More in detail, after 24 hours, it is possible to observe a dose-decrease on the CD83 surface marker (Figure 4.7 A). For the higher concentrations (1 and 10µg/mL) this decrease is comparable to the control DEX. While, after 72 hours of treatment the modulation of the marker CD83 is not present (Figure 4.7 B).

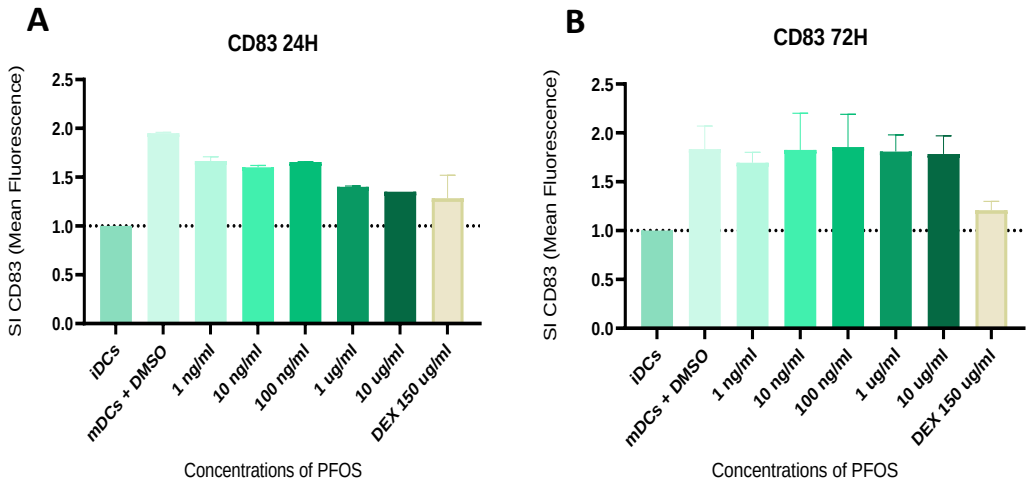


Figure 4.7 – CD83 expression following PFOS exposure. Immature DCs were exposed to increasing concentrations of PFOS and, 24 and 72 hours after the addition of the maturation cocktail, the CD83 expression was analyzed. The results are expressed as stimulation index (SI). The dotted line shown on the y-axis correspond to the control (SI=1).

Each value shown represents the mean ± standard error of the mean (SEM), with *n = 2* independent experiments.

As for the modulation of CD86 after 24 hours, the different concentrations of PFOS were not able to down modulate the expression of the marker like the positive control DEX (Figure 4.8 A). The same trend it is also observed after 72 hours (Figure 4.8 B).

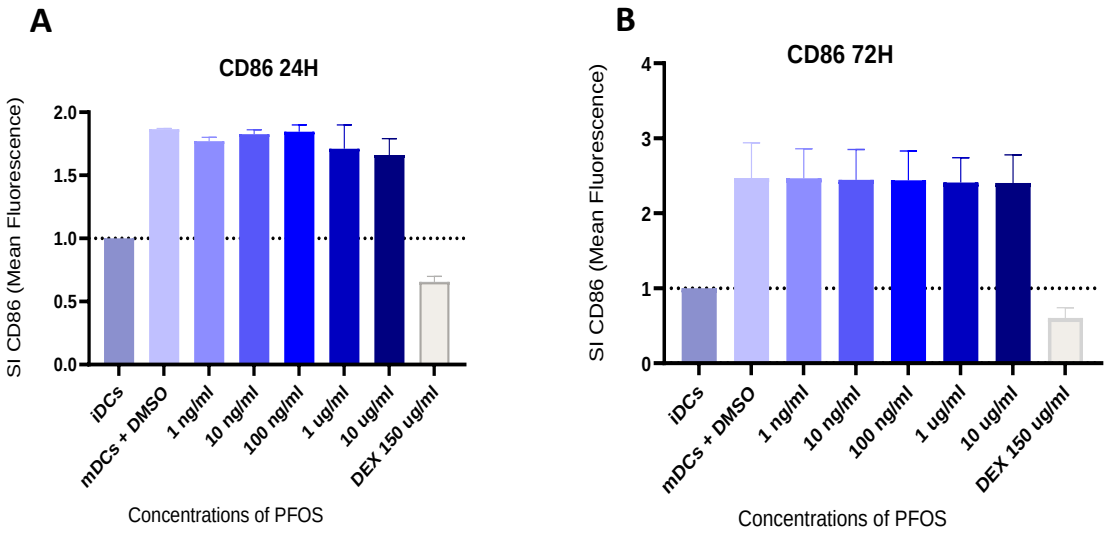


Figure 4.8 – CD86 expression following PFOS exposure. Immature DCs were exposed to increasing concentrations of PFOS and, 24 and 72 hours after the addition of the maturation cocktail, the CD86 expression was analyzed. The results are expressed as stimulation index (SI). The dotted line shown on the y-axis correspond to the control (SI=1). Each value shown represents the mean ± standard error of the mean (SEM), with *n = 2* independent experiments.

After 24 hours of treatment, a decrease in the expression of HLA-DR compared to the mDCs for all the different concentrations of PFOS is observed (Figure 4.9 A). However, this down modulation is not comparable with the positive control DEX. While, after 72 hours of treatment, it is observed a significant dose-decrease for all the concentration used. In particular, the concentration of 1 µg/mL and 10 µg/mL are similar with the positive control DEX (Figure 4.9 B).

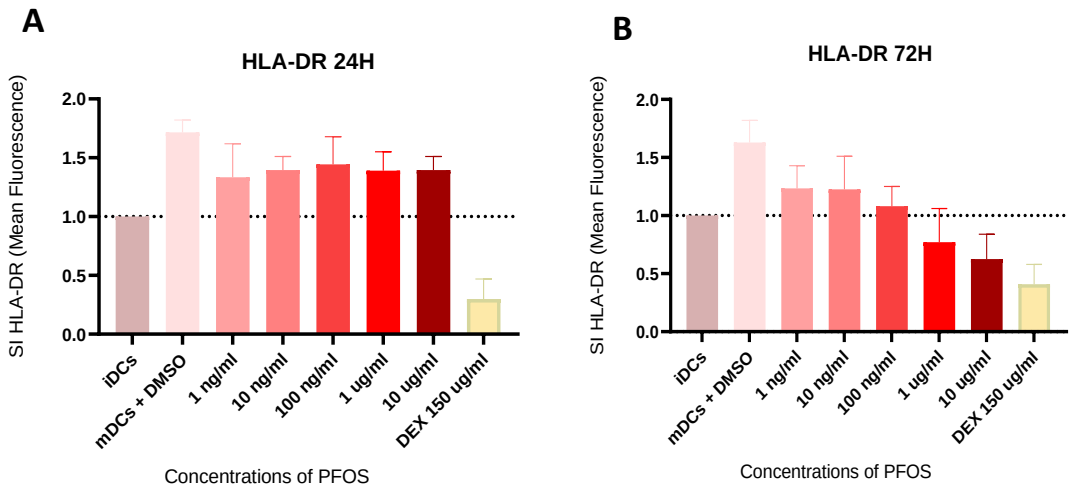


Figure 4.9 – HLA-DR expression following PFOS exposure. Immature DCs were exposed to increasing concentrations of PFOS and, 24 and 72 hours after the addition of the maturation cocktail, the HLA-DR expression was analyzed. The results are expressed as stimulation index (SI). The dotted line shown on the y-axis correspond to the control (SI=1). Each value shown represents the mean ± standard error of the mean (SEM), with *n = 2* independent experiments.

4.4 Discussion

To date, evidence based on epidemiological and laboratory studies supports that PFAS, such as PFOS and PFOA are presumed to be an immune hazard to humans. Despite the progress made in assessing the toxic effects of PFAS, there are still some gaps in the area of PFAS immunotoxicity as identified in the EFSA Scientific Opinion "Risk to human health related to the presence of perfluoroalkyl substances in food". Therefore, it is necessary to focus the research on the mechanisms underlying the observed immunosuppressive effects, mainly the reduction in the vaccination efficacy and the possible increase in the susceptibility to infectious disease, and secondly, on the assessment of a possible common mode of action and potency differences.

The chemical used in this work, PFOS, is known in literature to suppress multiple aspects of the immune system adding to the overall confidence that PFOS alters immune function in humans. Due to the different components of the immune system that may be targeted by PFOS, it is important to detect the changes of the human body in the level of cells or molecules, which could provide insight on possible biomarkers for PFOS-induced toxicity. However, the already available data about PFOS allows its use as reference PFAS which might provide insight into the relative potencies of relevant PFAS.

The aim of this project was to evaluate the immunotoxic effects of PFOS *in vitro* and specifically regarding the effect of it on cytokines-cocktail induced maturation of DCs. This experiment involved inducing the differentiation of THP-1 promyelocytic cells into iDCs and then treating the latter with the chemical for 24 hours. The maturation cocktail was added next in order to obtain mDCs and changes in the expression of the surface markers CD83, CD86 and HLA-DR after 24 and 72 hours of treatment.

The results obtained in this work demonstrate that the maturation of DCs was obtained as the expression of all the markers was induced for the mDCs compared with the iDCs. Furthermore, the results were compared to the positive control, DEX. The results on the expression of CD83 show a dose-decrease after 24 hours which for the concentrations 1 and 10μg/mL is comparable to DEX, but the same down-regulation of the marker is not observed after 72 hours of treatment. The expression of CD86 was not significantly different from the control at any of the *in vitro* exposure concentrations for PFOS neither after 24 hours nor after 72 hours. As for HLA-DR, a significant dose-decrease was observed for all the concentration used and particularly for the concentration of 1 μg/mL and 10 μg/mL after 72 hours of treatment.

Generally, the down-modulation of CD83 and HLA-DR might suggest that there is an immunosuppressive effect on the maturation of the DCs caused by PFOS but additional experiments are needed to confirm the results.

CONCLUSIONS

This thesis focused the studies on the *in vitro* evaluation of the immunotoxic effects of PFOS specifically on the maturation of DCs. In the first section, a literature review was performed to check the different manifestations of the immunotoxic effects of PFOS on the immune system. PFOS belongs to a large class of highly persistent chemicals, called PFAS, which were integrated in many industrial processes and consumer products because of their unique properties. Exposure to PFOS has been associated with adverse health effects on humans. A literature search on the toxicological evaluation of PFOS (National Toxicology Program, 2016), concluded that its assessment regarding immunotoxic effects relies for the most part on epidemiological and animal models. However, there are still some gaps that need to be covered mainly related to the mechanisms underlying the observed immunosuppressive effects and the assessment of a possible common mode of action and potency differences. By detecting the changes of the human body in the level of cells or molecules after PFOS exposure, it might be possible to understand better its mode of action or provide insight on possible biomarkers for PFOS-induced toxicity.

Based on the *in vivo* evidence of PFOS-induced immunosuppression, suitable *in vitro* models can be identified that allow the evaluation of parameters relevant for the toxicity *in vivo*, with particular attention to models reflecting antibody production. DCs can be found in various tissues, where they process antigens for presentation to T cells, establishing a link between innate and adaptive immune responses. Upon antigen recognition, DCs undergo a process of maturation characterized by conformational changes and the expression of co-stimulatory molecules (CD83, CD86) and increased levels of MHC. The functions of DCs have been extensively studied and their important role in immunity has led researchers to develop *in vitro* assays that focus on their functionalities to study immunotoxic effects from xenobiotics, using established cell lines that resemble their action. The promyelocytic THP-1 cell line, which is capable to differentiate into mDCs, has been extensively used for this aspect.

For the purposes of this experiment, THP-1 cells were differentiated into iDCs and the latter were treated with the chemical for 24 hours, then the maturation cocktail was added in order to obtain mDCs. Changes in the expression of CD83, CD86 and HLA-DR were evaluated by flow cytometry, 24 and 72 hours after the treatment. The results obtained suggest that PFOS might have an immunosuppressive effect on the maturation of DCs as there was a

down regulation on the expression of the marker CD83 and especially on HLA-DR. However, more investigation is needed to control if these results are statistically significant.

In the future, more PFAS should be tested to allow the detection of a possible common mode of action and potency differences. Nevertheless, the results obtained can be used, when confirmed, as a reference to provide insight into the relative potencies of relevant PFAS.

References:

- Aiba S, Terunuma A, Manome H, Tagami H. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. Eur J Immunol. 1997 Nov;27(11):3031-8. doi: 10.1002/eji.1830271141. PMID: 9394834.
- Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998 Mar 19;392(6673):245-52. doi: 10.1038/32588. PMID: 9521319.
- Bárcena J, Blanco E. Design of novel vaccines based on virus-like particles or chimeric virions. SubcellBiochem. 2013;68:631-65. doi: 10.1007/978-94-007-6552-8_21. PMID: 23737067.
- Berges C, Naujokat C, Tinapp S, Wieczorek H, Höh A, Sadeghi M, Opelz G, Daniel V. A cell line model for the differentiation of human dendritic cells. BiochemBiophys Res Commun. 2005 Aug 5;333(3):896-907. doi: 10.1016/j.bbrc.2005.05.171. PMID: 15963458.
- Bonifaz L., Bonnyay D., Mahnke K., Rivera M., Nussenzweig M.C. , Steinman R.M. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell toleranceJ. Exp. Med., 196 (2002), pp. 1627-1638
- Brase, Richard A et al. “Legacy and Emerging Per- and Polyfluoroalkyl Substances: Analytical Techniques, Environmental Fate, and Health Effects.” International journal of molecular sciences vol. 22,3 995. 20 Jan. 2021, doi:10.3390/ijms22030995
- Buser MC, Scinicariello F. Perfluoroalkyl substances and food allergies in adolescents. Environ Int. 2016;88:74–9.
- Cao W, Lee SH, Lu J. CD83 is preformed inside monocytes, macrophages and dendritic cells, but it is only stably expressed on activated dendritic cells. Biochem J. 2005 Jan 1;385(Pt 1):85-93. doi: 10.1042/BJ20040741. PMID: 15320871; PMCID: PMC1134676.
- Chaplin DD. Overview of the immune response. J Allergy ClinImmunol. 2010 Feb;125(2 Suppl 2):S3-23. doi: 10.1016/j.jaci.2009.12.980. PMID: 20176265; PMCID: PMC2923430.
- Christopher Lau, Katherine Anitole, Colette Hodes, David Lai, Andrea Pfahles-Hutchens, Jennifer Seed, Perfluoroalkyl Acids: A Review of Monitoring and Toxicological Findings, Toxicological Sciences, Volume 99, Issue 2, October 2007, Pages 366–394, https://doi.org/10.1093/toxsci/kfm128
- Coombs, P.R. and Gell, P.G. (1968) Classification of Allergic Reactions Responsible for Clinical Hypersensitivity and Disease. In: Gell, R.R., Ed., Clinical Aspects of Immunology, Oxford University Press, Oxford, 575-596.
- Corsini E, Avogadro A, Galbiati V, dell'Agli M, Marinovich M, Galli CL, Germolec DR. In vitro evaluation of the immunotoxic potential of perfluorinated compounds (PFCs). ToxicolApplPharmacol. 2011 Jan 15;250(2):108-16. doi: 10.1016/j.taap.2010.11.004. Epub 2010 Nov 12. PMID: 21075133.
- Corsini E, Sangiovanni E, Avogadro A, Galbiati V, Viviani B, Marinovich M, Galli CL, Dell'Agli M, Germolec DR. In vitro characterization of the immunotoxic potential of several perfluorinated compounds (PFCs). ToxicolApplPharmacol. 2012 Jan 15;258(2):248-55. doi: 10.1016/j.taap.2011.11.004. Epub 2011 Nov 18. PMID: 22119708.
- Corsini, E., Galbiati, V., Esser, P.R. et al. Role of PKC-β in chemical allergen-induced CD86 expression and IL-8 release in THP-1 cells. Arch Toxicol 88, 415–424 (2014). https://doi.org/10.1007/s00204-013-1144-z
- Cousins, Ian T et al. “The high persistence of PFAS is sufficient for their management as a chemical class.” Environmental science. Processes & impacts vol. 22,12 (2020): 2307-2312. doi:10.1039/d0em00355g
- Cousins, Ian T et al. “Strategies for grouping per- and polyfluoroalkyl substances (PFAS) to protect human and environmental health.” Environmental science. Processes & impacts vol. 22,7 (2020): 1444-1460. doi:10.1039/d0em00147c
- Dalsager L, Christensen N, Halekoh U, Timmermann CAG, Nielsen F, Kyhl HB, Husby S, Grandjean P, Jensen TK, Andersen HR. Exposure to perfluoroalkyl substances during fetal life and hospitalization for infectious disease in childhood: A study among 1,503 children from the Odense Child Cohort. Environ Int. 2021 Apr;149:106395. doi: 10.1016/j.envint.2021.106395. Epub 2021 Jan 25. PMID: 33508532..
- DeWitt JC, Blossom SJ, Schaider LA. Exposure to per-fluoroalkyl and polyfluoroalkyl substances leads to immunotoxicity: epidemiological and toxicological evidence. J Expo Sci Environ Epidemiol. 2019 Mar;29(2):148-156. doi: 10.1038/s41370-018-0097-y. Epub 2018 Nov 27. PMID: 30482935; PMCID: PMC6380927.

- Dong G-H, Zhang Y-H, Zheng L, Liu W, Jin Y-H, He Q-C. Chronic effects of perfluorooctanesulfonate exposure on immunotoxicity in adult male C57BL/6 mice. Arch Toxicol. 2009;83(9):805–15.
- Dong G-H, Liu M-M, Wang D, Zheng L, Liang Z-F, Jin Y-H. Sub-chronic effect of perfluorooctanesulfonate (PFOS) on the balance of type 1 and type 2 cytokine in adult C57BL6 mice. Arch Toxicol. 2011;85(10):1235–44.
- Elks J (14 November 2014). The Dictionary of Drugs: Chemical Data: Chemical Data, Structures and Bibliographies. Springer. pp. 366–. ISBN 978-1-4757-2085-3.
- Fair PA, Driscoll E, Mollenhauer MA, Bradshaw SG, Yun SH, Kannan K, Bossart GD, Keil DE, Peden-Adams MM. Effects of environmentally-relevant levels of perfluorooctanesulfonate on clinical parameters and immunological functions in B6C3F1 mice. J Immunotoxicol. 2011 Jan-Mar;8(1):17-29. doi: 10.3109/1547691X.2010.527868. Epub 2011 Jan 24. PMID: 21261439; PMCID: PMC3446244.
- Fenton, Suzanne E et al. “Per- and Polyfluoroalkyl Substance Toxicity and Human Health Review: Current State of Knowledge and Strategies for Informing Future Research.” Environmental toxicology and chemistry vol. 40,3 (2021): 606-630. doi:10.1002/etc.4890
- Galbiati V, Maddalon A, Iulini M, Marinovich M, Corsini E. Human keratinocytes and monocytes co-culture cell system: An important contribution for the study of moderate and weak sensitizers. Toxicol In Vitro. 2020 Oct;68:104929. doi: 10.1016/j.tiv.2020.104929. Epub 2020 Jul 3. PMID: 32628993.
- Galbiati V, Marinovich M, Corsini E. Mechanistic understanding of dendritic cell activation in skin sensitization: additional evidences to support potency classification. Toxicol Lett. 2020 Apr 1;322:50-57. doi: 10.1016/j.toxlet.2020.01.014. Epub 2020 Jan 17. PMID: 31958493.
- Grandjean, Philippe and EsbenBudtz-Jørgensen. “Immunotoxicity of perfluorinated alkylates: calculation of benchmark doses based on serum concentrations in children.” Environmental Health 12 (2013): 35 - 35.
- Glüge, Juliane et al. “An overview of the uses of per- and polyfluoroalkyl substances (PFAS).” Environmental science. Processes & impacts vol. 22,12 (2020): 2345-2373. doi:10.1039/d0em00291g.
- Gough SC, Simmonds MJ. The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action. Curr Genomics. 2007 Nov;8(7):453-65. doi: 10.2174/138920207783591690. PMID: 19412418; PMCID: PMC2647156.
- Granum B, Haug LS, Namork E, Stølevik SB, Thomsen C, Aaberge IS, van Loveren H, Løvik M, Nygaard UC. Pre-natal exposure to perfluoroalkyl substances may be associated with altered vaccine antibody levels and immune-related health outcomes in early childhood. J Immunotoxicol. 2013 Oct-Dec;10(4):373-9. doi: 10.3109/1547691X.2012.755580. Epub 2013 Jan 25. PMID: 23350954.
- Grosche L, Knippertz I, König C, Royzman D, Wild AB, Zinser E, Sticht H, Muller YA, Steinkasserer A, Lechmann M. The CD83 Molecule - An Important Immune Checkpoint. Front Immunol. 2020 Apr 17;11:721. doi: 10.3389/fimmu.2020.00721. PMID: 32362900; PMCID: PMC7181454.
- Guruge KS, Hikono H, Shimada N, Murakami K, Hasegawa J, Yeung LW, Yamanaka N, Yamashita N. Effect of perfluorooctanesulfonate (PFOS) on influenza A virus-induced mortality in female B6C3F1 mice. J Toxicol Sci. 2009 Dec;34(6):687-91. doi: 10.2131/jts.34.687. PMID: 19952504.
- Hubo M, Trinschek B, Kryczanowsky F, Tuettenberg A, Steinbrink K, Jonuleit H. Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. Front Immunol. 2013 Apr 3;4:82. doi: 10.3389/fimmu.2013.00082. PMID: 23565116; PMCID: PMC3615188.
- Humblet O, Diaz-Ramirez LG, Balmes JR, Pinney SM, Hiatt RA. Perfluoroalkyl chemicals and asthma among children 12–19 years of age: NHANES (1999–2008). Environ Health Perspect. 2014;122(10):1129–33.
- Huston DP. The biology of the immune system. JAMA. 1997 Dec 10;278(22):1804-14. PMID: 9396641.
- Iulini M, Maddalon A, Galbiati V, Marinovich M, Corsini E. In vitro identification of drugs inducing systemic hypersensitivity reactions known in vivo to be associated with specific HLA genotypes.Toxicol In Vitro. 2020 Oct;68:104953. doi: 10.1016/j.tiv.2020.104953. Epub 2020 Jul 28. PMID: 32730864.
- Janeway CA Jr, Bottomly K, Horowitz J, Kaye J, Jones B, Tite J. Modes of cell:cell communication in the immune system. J Immunol. 1985 Aug;135(2 Suppl):739s-742s. PMID: 3159798.
- Johnson, Mark S et al. “Estimating Environmental Hazard and Risks from Exposure to Per- and Polyfluoroalkyl Substances (PFASs): Outcome of a SETAC Focused Topic Meeting.” Environmental toxicology and chemistry vol. 40,3 (2021): 543-549. doi:10.1002/etc.4784
- Kielsen K, Shamim Z, Ryder LP, Nielsen F, Grandjean P, Budtz-Jørgensen E, Heilmann C. Antibody response to booster vaccination with tetanus and diphtheria in adults exposed to perfluorinated alkylates. J Immunotoxicol. 2016;13(2):270-3. doi: 10.3109/1547691X.2015.1067259. Epub 2015 Jul 16. PMID: 26181512; PMCID: PMC4739630.

- H.K. Knutsen, J. Alexander, L. Barregård, M. Bignami, B. Brüschweiler, S. Ceccatelli, et al., Risk to human health related to the presence of perfluorooctane sulfonic acid and perfluorooctanoic acid in food, EFSA J., 16 (2018), 10.2903/j.efsa.2018.5194
- Ladics GS. The Sheep Erythrocyte T-Dependent Antibody Response (TDAR) In: Methods in molecular biology (Clifton, NJ:). 2018. p. 83–94.
- Larosa DF, Orange JS. 1. Lymphocytes. J Allergy ClinImmunol. 2008 Feb;121(2 Suppl):S364-9; quiz S412. doi: 10.1016/j.jaci.2007.06.016. PMID: 18241683.
- Lefebvre DE, Curran I, Armstrong C, Coady L, Parenteau M, Liston V, et al. Immunomodulatory effects of dietary potassium perfluorooctanesulfonate (PFOS) exposure in adult Sprague-Dawley rats. J Toxicol Environ Health A. 2008;71(23):1516–25.
- Li Z, Ju X, Silveira PA, Abadir E, Hsu WH, Hart DNJ, Clark GJ. CD83: Activation Marker for Antigen Presenting Cells and Its Therapeutic Potential. Front Immunol. 2019 Jun 7;10:1312. doi: 10.3389/fimmu.2019.01312. PMID: 31231400; PMCID: PMC6568190.
- Looker C, Luster MI, Calafat AM, Johnson VJ, Burleson GR, Burleson FG, et al. Influenza vaccine response in adults exposed to perfluorooctanoate and perfluorooctanesulfonate. Toxicol Sci. 2014;138(1):76–88
- Lv QY, Wan B, Guo LH, Yang Y, Ren XM, Zhang H. In vivo immunotoxicity of perfluorooctanesulfonate in BALB/c mice: Identification of T-cell receptor and calcium-mediated signaling pathway disruption through gene expression profiling of the spleen. ChemBiol Interact. 2015 Oct 5;240:84-93. doi: 10.1016/j.cbi.2015.07.015. Epub 2015 Aug 20. PMID: 26300304.
- Ma DY, Clark EA. The role of CD40 and CD154/CD40L in dendritic cells. SeminImmunol. 2009 Oct;21(5):265-72. doi: 10.1016/j.smim.2009.05.010. Epub 2009 Jun 12. Erratum in: SeminImmunol. 2010 Jun;22(3):190. PMID: 19524453; PMCID: PMC2749083.
- Manzoor A Mir (2013) Costimulation and costimulatorymolecules. ISBN No. 978-3-659-39067-8; Book Title: Cancerand Infectious Diseases; Chapter-1, Page No.s 1-46; LAPPublishers Germany
- Matasić R, Dietz AB, Vuk-Pavlović S. Dexamethasone inhibits dendritic cell maturation by redirecting differentiation of a subset of cells. J Leukoc Biol. 1999 Dec;66(6):909-14. doi: 10.1002/jlb.66.6.909. PMID: 10614771.
- K. Midgett, M.M. Peden-Adams, G.S. Gilkeson, D.L. Kamen In vitro evaluation of the effects of perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) on IL-2 production in human T-cells J. Appl. Toxicol., 35 (2015), pp. 459-465
- Mogensen UB, Grandjean P, Heilmann C, Nielsen F, Weihe P, Budtz-Jorgensen E. Structural equation modeling of immunotoxicity associated with exposure to perfluorinated alkylates Children's Environmental Health. Environ Heal A Glob Access Sci Source. 2015;14(1):1–10.
- Mollenhauer MA, Bradshaw SG, Fair PA, McGuinn WD, Peden-Adams MM. Effects of perfluorooctanesulfonate (PFOS) exposure on markers of inflammation in female B6C3F1 mice. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2011;46(2):97-108. doi: 10.1080/10934529.2011.532418. PMID: 21170772.
- National Center for Biotechnology Information. PubChem Compound Summary for CID 74483, Perfluorooctanesulfonic acid. https://pubchem.ncbi.nlm.nih.gov/compound/Perfluorooctanesulfonic-acid. Accessed July 7, 2022.
- National Center for Biotechnology Information. "PubChem Compound Summary for CID 5743, Dexamethasone" PubChem, https://pubchem.ncbi.nlm.nih.gov/compound/Dexamethasone#section=Evaluations-of-the-Joint-FAO-WHO-Expert-Committee-on-Food-Additives---JECFA. Accessed 8 July, 2022.
- Ng Carla et al. "Addressing Urgent Questions for PFAS in the 21st Century." Environmental science & technology vol. 55,19 (2021): 12755-12765. doi:10.1021/acs.est.
- NTP (National Toxicology Program). 2016. Monograph on Immunotoxicity Associated with Exposure to Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS). Research Triangle Park, NC: National Toxicology Program. https://ntp.niehs.nih.gov/ntp/ohat/pfoa_pfos/pfoa_pfosmonograph_508.pdf
- OECD; Hazard Assessment of Perfluorooctane Sulfonate (PFOS) and its Salts. Paris, France: Organizat ion for EconomiCooperation and Development, Available from, as of November 22, 2010: https://www.o ecd.org/dataoecd/23/18/2382880.pdf
- Osuna CE, Grandjean P, Weihe P, El-Fawal HAN. Autoantibodies associated with prenatal and childhood exposure to environmental chemicals in Faroese children. Toxicol Sci. 2014;142(1):158–66.
- Peden-Adams MM, Keller JM, Eudaly JG, Berger J, Gilkeson GS, Keil DE. Suppression of humoral immunity in mice following exposure to perfluorooctanesulfonate. Toxicol Sci. 2008;104(1):144–54.

- Peyton Myers L Clinical Immunotoxicology In: Methods in molecular biology (Clifton, NJ:). 2018. p. 15–26.
- Pichler WJ. Delayed drug hypersensitivity reactions. Ann Intern Med. 2003 Oct 21;139(8):683-93.
- Piemonti L, Monti P, Allavena P, Sironi M, Soldini L, Leone BE, et al.Glucocorticoids Affect Human Dendritic Cell Differentiation andMaturation. J Immunol (1999) 162:6473–81.
- Qazi MR, Nelson BD, DePierre JW, Abedi-Valugerdi M. 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? Toxicology. 2010;267(1–3):132–9.
- S. Rayne, K. Forest e K. J. Friesen, Congener-specific numbering systems for the environmentally relevant C1 through C8 perfluorinated homologue groups of alkyl sulfonates, carboxylates, telomer alcohols, olefins, and acids, and their derivatives, in Journal of Environmental Science and Health, Part A, vol. 43, n. 12, 2008, pp. 1391-1401, DOI:10.1080/10934520802232030
- Rogers, R.D., Reh, C.M. &Breysse, P. Advancing per- and polyfluoroalkyl substances (PFAS) research: an overview of ATSDR and NCEH activities and recommendations. J Expo Sci Environ Epidemiol 31, 961–971 (2021). https://doi.org/10.1038/s41370-021-00316-6
- Rosato Isabellaet al. How to investigate human health effects related to exposure to mixtures of per- and polyfluoroalkyl substances: A systematic review of statistical methods, Environmental Research, Volume 205, 2022, 112565, ISSN 0013-9351, https://doi.org/10.1016/j.envres.2021.112565.
- D. Schrenk, M. Bignami, L. Bodin, J.K. Chipman, J. del Mazo, B. Grasl- Kraupp, et al., Risk to human health related to the presence of perfluoroalkyl substances in food, EFSA J., 18 (2020), 10.2903/j.efsa.2020.6223
- Stein CR, McGovern KJ, Pajak AM, Maglione PJ, Wolff MS. Perfluoroalkyl and Polyfluoroalkyl Substances and Indicators of Immune Function in Children Aged 12 – 19 years: National Health and Nutrition Examination Survey. Pediatr Res. 2015;79(2):348–57.
- Vetvicka V, Vetvickova J. Reversal of perfluorooctanesulfonate-induced immunotoxicity by a glucan-resveratrol-vitamin C combination. Orient Pharm Exp Med. 2013;13(1):77–84.
- Wang C, Sun W, Ye Y, Bomba HN, Gu Z. Bioengineering of Artificial Antigen Presenting Cells and Lymphoid Organs. Theranostics. 2017 Aug 17;7(14):3504-3516. doi: 10.7150/thno.19017. PMID: 28912891; PMCID: PMC5596439.
- Wang Z, DeWitt JC, Higgins CP, Cousins IT. A Never-Ending Story of Per- and Polyfluoroalkyl Substances (PFASs)? Environ Sci Technol. 2017;51(5):2508–18.
- Wu HJ, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes. 2012 Jan-Feb;3(1):4-14. doi: 10.4161/gmic.19320. Epub 2012 Jan 1. PMID: 22356853; PMCID: PMC3337124.
- Yamaki K, Yoshino S. Enhancement of FcεRI-mediated degranulation response in the rat basophilic leukemia cell line RBL2H3 by the fluorosurfactantsperfluorooctanoic acid and perfluorooctanesulfonate. Environ ToxicolPharmacol. 2010 Mar;29(2):183-9. doi: 10.1016/j.etap.2009.12.009. Epub 2010 Jan 7. PMID: 21787600.
- Zeng Z, Song B, Xiao R, Zeng G, Gong J, Chen M, Xu P, Zhang P, Shen M, Yi H. Assessing the human health risks of perfluorooctanesulfonate by in vivo and in vitro studies. Environ Int. 2019 May;126:598-610. doi: 10.1016/j.envint.2019.03.002. Epub 2019 Mar 8. PMID: 30856447.
- Zheng L, Dong GH, Jin YH, He QC. Immunotoxic changes associated with a 7-day oral exposure to perfluorooctanesulfonate (PFOS) in adult male C57BL/6 mice. Arch Toxicol. 2009;83(7):679–89.
- Zhou LJ, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J Immunol. (1995) 154:3821–35.