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PAPEL DE LOS LINFOCITOS T CD4 Y CD8, B Y CELULAS NK EN LA ONCOVIGILANCIA DEL LOBO MARINO DE CALIFORNIA: UNA APROXIMACIÓN ECOTOXICOLÓGICA

TESIS INDIVIDUAL

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Resumen

En las últimas décadas, el número de casos de carcinoma de origen urogenital en el lobo marino de California (Zalophus californianus) ha aumentado hasta llegar a una prevalencia de 26% en individuos varados en la costa oeste de EEUU. En otras áreas de la distribución de la especie, como las islas del Pacífico Norte de México y el Golfo de California se han encontrado transformaciones tisulares en el epitelio genital pero no se han reportado casos de carcinoma urogenital. Uno de los factores de riesgo asociados a este cáncer son la exposición a altos niveles de organoclorados (PCBs y DDTs), mismos que difieren marcadamente entre las regiones. Estos contaminantes pueden asociarse con la oncogénesis de manera directa, o indirecta, al suprimir a las poblaciones celulares de oncovigilancia, como los linfocitos T citotóxicos y células NK, así como a células de apoyo como linfocitos T +CD4 y T-reguladoras. En esta tesis se investigó el efecto de los organoclorados a partir de ensayos in vitro de proliferación y mitogénesis de linfocitos aislados de nódulos linfáticos de lobos marinos varados, y de la cuantificación ex vivo de los transcritos de las subpoblaciones inmunes sanguíneas de lobos marinos aparentemente sanos, provenientes de diferentes zonas geográficas. Se determinó que los congéneres de PCB tipo dioxina 105 y 138, a altas concentraciones ocasionaron una reducción en la proliferación linfocitaria, mientras que los congéneres tipo no dioxina 138, 153 y 180 a bajas concentraciones tuvieron un efecto linfoproliferativo. Algunos genes, como GATA3 y STAT-1, incrementaron su transcripción en las poblaciones del lobo marino de la California con un patrón latitudinal, mostrando niveles de transcripción similar en la región comprendida por las islas del Golfo San Esteban, San Pedro Nolasco y San Pedro Mártir con respecto a la registrada para el archipiélago del Pacífico San Benito, en el Pacífico Norte Mexicano. No se encontró evidencia de una asociación entre los niveles de transcripción leucocitaria circulante y la transformación epitelial pre-cancerígena, aunque se sugiere una relación ligera entre los coilocitos y las células T reguladoras, que es típica respuesta frente a papilomavirus. Este estudio constituye un primer paso para comprender el efecto funcional de los organoclorados sobre las células oncovigilantes en relación al proceso de transformación del epitelio genital del lobo marino de California.

Palabras clave: Linfocitos T, células NK, oncovigilancia, organoclorados, lobo marino de California, *Zalophus californianus*

Summary

The number of cases of urogenital carcinoma in the California sea lion (Zalophus californianus) has increased markedly in the past decades, reaching 26% of prevalence in individuals stranded along the western coast of the US. In other areas of their distribution, such as the Mexican northern Pacific and Gulf of California, pre-cancerous transformation of the genital epithelium has been found, but there have been no reported cases of urogenital carcinoma. One of the risk factors associated with this type of cancer is exposure to high levels of organochlorine pesticides (PCBs y DDTs), which vary markedly in concentration among regions. These pollutants can be directly and indirectly associated with occurrence of cancer, be it by inducing carcinogenesis or affecting oncovigilant cells such as cytotoxic T-lymphocytes and NK cells, as well as helper cells such as T-CD4 lymphocytes and T-regulators. This thesis investigated the effect of organochlorines by using *in vitro* PCB exposure assays that quantified proliferation and mitogenesis of lymphocytes harvested from peripheral lymph nodes from stranded adult sea lions; furthermore, I used *ex vivo* quantitation of transcription levels of key genes involved in oncovigilance in blood collected from apparently healthy adult sea lions sampled in different geographic zones. My results showed that dioxin-like PCB congeners 105 and 138, when at high concentrations, led to a decrease in proliferation, while non-dioxin-like congeners 138, 153 y 180, when at low concentrations caused proliferation. Some genes, such as GATA3 and STAT-1, varied their transcription levels with a spatial pattern; levels were similar in colonies found in the Midriff region and eastern-central region, and different to those of the colony located in the Mexican northern Pacific. There was no evidence that transcription levels and precancerous transformation of the genital epithelium were associated, except for a suggested relationship between koilocytes and Treg activity, which is typical of papillomavirus infections. This study is a first step towards understanding the functional effect of organochlorine contaminants on oncovigilant cells in the context of epithelial transformation of the California sea lion.

Key words: T cells, NK cells, organochlorines, oncovigilance, California sea lion, *Zalophus californianus*

Dedication

El trabajo de esta tesis no podría haber sido llevado a cabo sin el apoyo de mis compañeros de laboratorio, de mis amigos y de mi familia. Y, obviamente, sin el ambiente y recursos que ha aportado el laboratorio. Agradezco todos los momentos en los que me han brindado ayuda y me han dado fuerzas para continuar.

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Index

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

The California sea lion (CSL) is a social and gregarious otariid whose distribution encompasses the coastline and insular areas from British Columbia in Canada to the Mexican Pacific central coast. In the 1980's, reports of a highly aggressive type of carcinoma that involved the genital epithelium began to appear (Brown *et al*., 1980). Between 1979 and 1994 urogenital carcinoma was found in 18% of dead stranded adult sea lions that underwent post-mortem examinations (Gulland *et al*., 1996). The incidence has increased markedly, however, and is now the cause of death of 26% of adult sea lions stranded along the US Pacific coast (Browning *et al*., 2015). Interestingly, this is the only geographic area where urogenital carcinoma has been reported in the species to date, although in other areas of their distribution, such as the Gulf of California and Baja California (Mexico), precancerous transformation of the genital epithelium has been found, and appears to be relatively prevalent even in young animals (Barragán-Vargas *et al*., 2016). These regions are comparatively less-industrialized than the US Pacific coast.

Most types of cancer have a multifactorial etiology (e.g. Mutsaers *et al*., 2003; Haverkos, 2005), and the urogenital carcinoma of CSL is not an exception. To date, various risk factors have been identified, including inbreeding (Acevedo-Whitehouse *et al*., 2003a), specific alleles at different genes (Bowen *et al*., 2005; Browning *et al*., 2015), infection by the potentially-oncogenic otarine gamma herpesvirus, OtHV-1 (Lipscomb et al. 2000; Buckles et al. 2006, 2007), beta-haemolytic streptococci (Johnson *et al*., 2006), and high levels of persistent organic pollutants in the blubber (Ylitalo *et al*., 2005). However, a comparative analysis of these risk factors across CSL populations has yet to be conducted. Such an approach would allow us to discern the main triggers

of carcinogenesis in this species, and understand why some populations are affected by urogenital cancer and others appear not to be prone to develop this pathology (Barragán-Vargas *et al*., 2016). Specifically, this thesis aimed to investigate whether onco-surveillant lymphoid cell populations are related to transformation of the genital epithelium and to examine their potential role in carcinogenesis under exposure to organic pollutants.

Cytotoxic T lymphocytes (CTL), also known as $CD8^+$ lymphocytes, and NK cells are responsible for surveying and controlling precancerous cells before they develop malignancies, proliferate as a cancerous tumor, and in some cases metastasize (Hanahan and Weinberg 2000, 2011; Ruffell *et al*., 2010). In addition, these immune effectors destroy cells infected by intracellular parasites, such as viruses, which can also be tumorigenic. Both lymphocyte cell types act via the release of perforines and granzymes that break the cell membranes and induce apoptosis (Chiang *et al*., 2013), and the synthesis of tumoral necrosis factor (TNF), but they differ in their timing and in terms of other cytokines that they synthesize (Chiang *et al*., 2013). As NK cells do not need specific priming, they are quick to act and rely on a balance between inhibitor and activator molecules (Vivier *et al*., 2011). On the other hand, in order to act, CTL require antigen presentation by major histocompatibility complex (MHC) type I molecules expressed on affected cells, and activation by helper T lymphocytes (T-helper), also known as $CD4^+$ cells (Kindt *et al*., 2007; Vivier *et al*., 2011). Once CTL and NK cells detect a cellular abnormality, they eliminate the target cell. Based on their known mechanisms, it is parsimonious to hypothesize that both lymphocyte populations have important onco-surveillant and anti-viral roles that are key to avoid the development of urogenital carcinoma of the CSL.

NK and CTL cells are closely controlled by other cell populations, which regulate their activation and maturation to allow them to act in proportion to the level of cell damage.

Macrophages, T-helper cells (DeNardo *et al*., 2010; Ruffell *et al*., 2010) and T regulator lymphocytes (Treg) are the main cellular effectors that modulate the action of these cytotoxic cells (Beyer and Schultze 2006; DeNardo *et al*., 2010; Ruffell *et al*., 2010). T-helper and Treg lymphocytes produce specific cytokines that intensify the activity of NK and CTL cells or drive them towards a 'tolerance status' that avoids autoimmune processes. This balance is indispensable for the organisms to have an adequate response, one that will not compromise the organism's health (DeNardo *et al*., 2010; Ruffell *et al*., 2010).

Under environmental pressures, such as pollutants, it has been shown that an organism can suffer various health alterations. Organochlorines, such as polychlorinated biphenyl compounds (PCBs) and dichlorodiphenyltrichloroethane (DDT), are persistent organic pollutants that tend to bioaccumulate in the food chains, especially in top predators that are long-lived and have thick blubber layers (Borrell *et al*., 2010). Under laboratory conditions, the direct carcinogenic role of organochlorines has been demonstrated (Silberhorn *et al*., 1990; Robertson and Hansen 2001). Furthermore, even when their concentrations are below the carcinogenic level, they can elicit immune toxicity and lead to immunomodulation (Beckmen *et al*., 1999, 2003; Levin *et al*., 2004, 2007; Mori *et al*., 2006, 2008; Desforges *et al*., 2016).

While there is still a lack of strong empirical evidence that persistent organic pollutants cause damage to exposed organisms in the wild, an environmental threshold for infectious diseases was proposed as 17 mg/kg of the sum of 25 common PCB congeners and has been proven for another marine mammal, the harbor porpoise, *Phocoena phocoena* (Jepson *et al*., 2005). Furthermore, a correlation between organochlorine levels and the development of urogenital carcinoma in CSL has already been proposed, as individuals with cancer tended to have higher levels of PCBs and DDTs (Ylitalo *et al*., 2005). Taking into account the recorded concentrations of organochlorines

in the organisms, that are below direct carcinogenic levels (Ylitalo *et al*., 2005), a causal relationship between organochlorines and urogenital carcinoma is probably based on an immunomodulatory effect rather than on direct carcinogenicity of the compounds. To date, there are no published studies that have focused on how NK and CTL cells are affected by organic pollutants in free-ranging marine mammals, although it has been studied under laboratory conditions (de Guise 1998; Mori *et al*., 2006, 2008). Due to their oncogenic surveillance role cytotoxic ability against tumorous cells, it is plausible to speculate that exposure to organochlorines affects their function negatively.

This thesis aimed to elucidate the role of NK, CTL, and other T cell populations involved with immunomodulation, on the transformation of the genital epithelium. The working hypotheses were: *i*) persistent organic pollutants exert an immunomodulative effect on CSL oncosurveillant immune cells, *ii*) immune profiles of CSL vary spatially, and *iii*) modulation of the immune system is related to the transformation of the genital epithelium. To challenge the first hypothesis, I used *in vitro* assays of proliferation and cytotoxicity of CSL NK and T cells under environmentally-comparable concentrations of PCB mixtures and DDT. For the second and third hypothesis, I quantified specific gene transcripts of oncosurveillant lymphoid populations and support cells collected from CSLs sampled at different breeding colonies in the Gulf of California, and Mexican Northern Pacific, and I analyzed transcription levels in relation to cell transformation of the genital epithelium.

2. BACKGROUND

2.1 Generalities of cancer

The term cancer encompasses several pathologies based on cellular malignancy and abnormal proliferation, which originate by genetic mutations, and epigenetic modifications. Cancer consists of an abnormal division of transformed cells that become able to invade the tissue where they originated, as well as adjacent and remote tissues (Wright, 2012). The survival of cancer cells depends on their capacity to hijack resources, to use the normal physiological process and resources for their own benefit (De Visser *et al*., 2006), and to promote their immortalization (Hanahan and Weinberg, 2000, 2011).

Lack of control of cell division can lead to the occurrence of cancer. However, cells have mechanisms to repair malfunction, namely by committing 'suicide' by apoptosis or by inducing senescence through modulation of the E2F signaling pathway (Andreu *et al*., 2005; Vesely *et al*., 2011). This is why, although cells continuously transform in the body, carcinogenesis does not always occur (Kim *et al*., 2007).

Although there are many hypotheses proposed to explain the occurrence of cancer, including accumulation of mutations (Vesely *et al*., 2011) in a Darwinian-like model (Alison *et al*., 2012), a mutator phenotype (Loeb *et al*., 2008), the presence of aneuploidy (Gordon *et al*., 2012), and cancer stem cells (Alison *et al*., 2012), when analyzed in detail all hypotheses converge in one of the aspects of the original theory of carcinogenesis: namely, at least two independent events are needed for carcinogenesis to occur. When both events happen, transformed cells abandon their normal function and instead focus only of their own persistence and proliferation, even being able

to invade other tissues via the blood stream, and, sometimes, lead to death by compromising the function of organs and systems. If only one event happens, transformed cells are still under homeostatic control of the body and undergo apoptosis, be it self-induced or caused by oncovigillant cells.

There are two frequent kinds of mutations associated with cancer: those that occur in proto-oncogenes and those that occur in tumor suppression genes (Gout and Huot, 2008; Levine and Puzio-Kuter, 2010). Proto-oncogenes are genes that under normal conditions rule over various cellular pathways, such as maturation and proliferation, and mostly remain silenced after early development. Viral infections, direct mutation, or mutations in their promotor can activate a proto-oncogen (Gout and Huot, 2008), thus leading to uncontrolled mitosis. In contrast, tumor suppression genes tend to be active, and their gene products are essential for arresting cell proliferation and, thus, govern the cell cycle. Tumor suppressor genes do not need to have two functional alleles, they can work with only one copy, but when both alleles are damaged there is a high risk for uncontrolled proliferation and cancer (Lodish *et al*., 2000). Some pathogens, such as oncogenic herpesvirus or papillomaviruses, carry oncogenes in their genome and can transcribe them once they have infected the target cell (Moore and Chang, 2010).

Hanahan and Weinberg (2003, 2011) propose that it is necessary for tumorigenic cells to acquire six traits in order for cancer to occur (Fig. 2.1): namely, they need to maintain a proliferation signal, they must avoid growth-suppressor signals, resist cell death, maintain immortality, induce angiogenesis, and invade other tissues. They also require the reorganization of normal metabolism in their favor, and need to develop defensive mechanisms against the oncovigilant immune cells such as NK and CTL (Vesely *et al*., 2011) (Fig. 2.2). Moreover, cancer

cells recruit other (normal) cells such as macrophages and fibroblasts (Barcellos-Hoff *et al.*, 2013) in order to build a microniche and use their traits to further the growth of these abnormal cells.

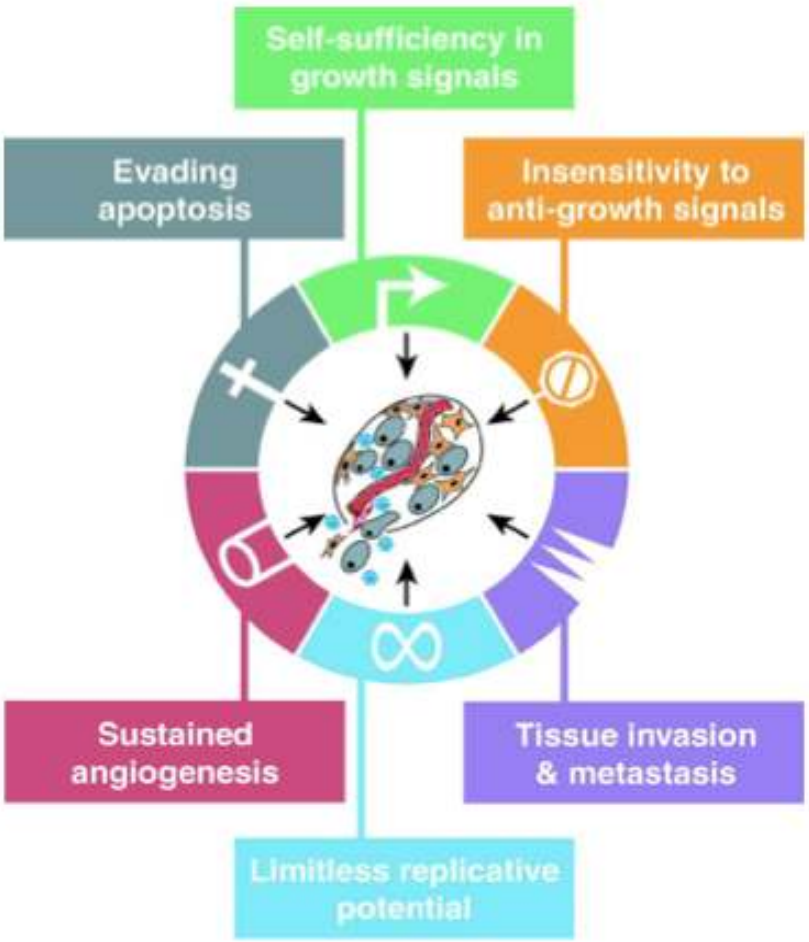

Figure 2.1 The six hallmarks of cancer (from Hanahan and Weinberg, 2000).

2.1.1 Development of cancer

Certain models of cancer progression fit some types of cancers but there is still no 'universal model' to suit all kinds of cancer. As development of urogenital carcinoma is not yet understood

for the CSL, a simplified model would be the best approach to attempt to study it. Cancer begins with the transformation of one cell. The transformation is just one of the hallmarks of cancer, and it is an insufficient condition. If unchecked, this status can lead to neoplasia, that can be benign if its development is limited and there are no signs of malignancy, or malignant. Malignancy is dependent of the cancer and cell type, and usually is characterized by the size of the tumor and differences in the rate of mitosis, or malignant (cancerous) if it is not. If malignant, the neoplasia can eventually invade other tissues; a process known as metastasis. Thus, transformation is the starting step that drives, or fails to do so, tumor formation (Yamauchi and Fujita, 2012). In this step, a normal cell, be it a stem cell or a differentiated cell, will acquire a new status, that of a 'cancer stem cell', with unrestricted proliferation and the possibility of originating a new kind of cellular heterogeneity: tumor heterogeneity (Guo *et al*., 2006; Alison *et al.*, 2012; Marshall *et al.*, 2014).

Cancer is initially an *in situ* malignant neoplasia that is named in terms of the site of origin. Muscular or connective tissue neoplasias are termed sarcomas; those that occur in the blood cells are leukemias; in lymphoid tissue they are termed lymphomas; in nervous cells they are known as neuroblastoma and, finally, those that start in epithelia are denominated carcinomas (Muñoz, 1997). Once a neoplasia has started, proliferation is sustained although the originating causes may have disappeared. All neoplasias have the potential to expand locally and often do so aggressively. Its expansion can occur via the invasion of the interstitium, the lymphatic ducts, the blood stream, caelomic cavities, aponeurosis and fascia-associated spaces, cerebrospinal fluid, and through epithelial cavities (Kaiser, 1989). However, neoplasia expansion produces less potential harm to the animal due to its limited expansion capability, particularly in comparison with the damage caused by metastasis (Kaiser, 1989).

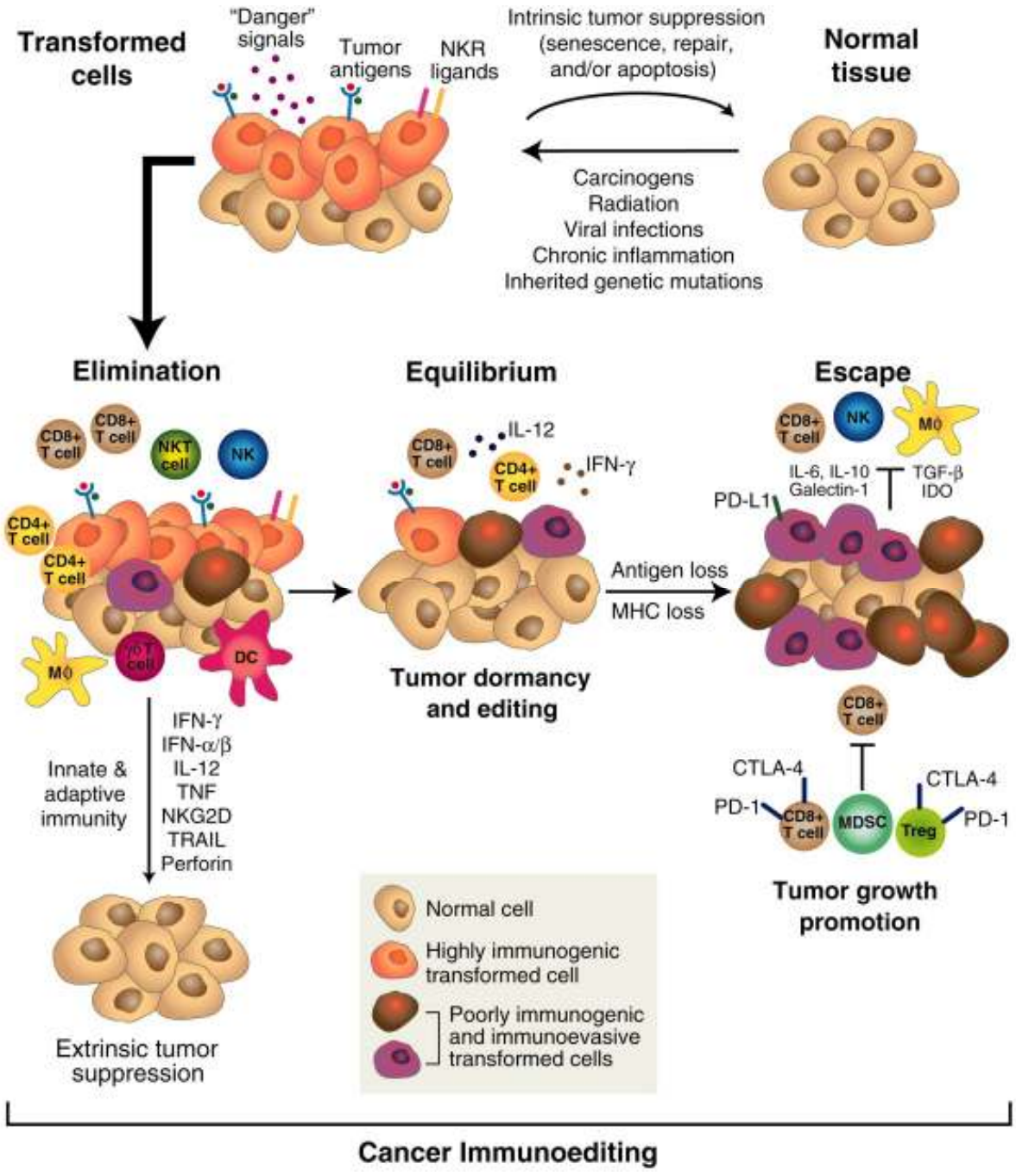

Figure 2.2 Cancer immunoediting (from Schreiber et al., 2011).

The second step in cancer development is the tumor progression. In this phase, the tumor acquires new characteristics, which make it dangerous for the hostage. At the beginning, every cell in the tumor is identical but, as it expands, they become different, a process called tumorheterogeneity (Cullen *et al.,* 1978). Until recently, alterations of the immune system were not commonly included among the events that can lead to cancer (Hanahan and Weinberg, 2011) but many authors had already started to consider the importance of a balance between protumoral and antitumoral activity of the cells involved (Kim *et al.*, 2007; Vesely *et al.*, 2011). Due to that, some authors (e.g. Kim *et al.*, 2007; Vesely *et al.*, 2011) have classified cancer stages according to the cancer-immune system interaction; namely elimination, equilibrium, and escape. Elimination consists of the recovery of the tissue, which acquires all of its original characteristics and returns to a non-tumorigenic status. Equilibrium occurs when the immune system halts tumor progression but is unable to eliminate all the cancerous cells so the tumor persists, albeit in a controlled state. Finally, escape entails victory of the tumor against the immune system and, thus, uncontrolled proliferation. However, stages do not progress in a linear fashion. Rather, the three are interconnected and depend on environmental pressures to progress between phases.

Malignant neoplasias can invade other tissues, in contrast to benign tumors, and they can form secondary nuclei whereas benign tumors are usually encapsulated or, at least, limited in their expansion. The secondary nuclei formation in other parts of the body is known as metastasis (Muñoz, 1997; Curran and Murray, 1999). In general, tumorigenic cells expand in a tissue until they are blocked by a barrier, such as the basal membrane, although some cancer cells are able to dissolve these blockages using enzymes and, thus, continue their expansion (Curran and Murray, 1999). The secondary nuclei are established in three ways: mostly, the blood and lymphoid vascular systems allow their establishment far from their original site (Cullen *et al*., 1978; Bravo-

Cordero *et al*., 2012) whereas trans-caelomic metastasis establishes nuclei closer to the original site (Cullen *et al*., 1978), and some nuclei (mainly carcinomas) form invadopodia, a structure that allows them to digest vascular basal walls and invade the blood vessels, finally, allowing transportation to other organs (Bravo-Cordero *et al*., 2012). From a clinical perspective, most damage derived from cancer is tissue invasion, and a substantial part of cancer-associated mortality results from metastasis (Curran and Murray, 1999; Bravo-Cordero *et al*., 2012).

2.1.2 Cancer in wildlife

It is generally thought that cancer affects mainly humans and domestic or laboratory animals, and there has not been much concern about cancer in wildlife until recently (McAloose and Newton, 2009). Although there have been various case reports of different neoplasias in wild animals before the 80's (Clark, 1973), it was not until that decade that the number of reports increased, as did their perceived importance. Currently, cancer is considered to be able to reduce reproduction rates, affect population dynamics, and lead to population declines (McCallum, 2006; McAloose and Newton, 2009). Perhaps the most representative case of wildlife cancer as a cause of population decline is the contagious facial cancer of the Tasmanian devil, *Sarcophilus harrisii*, as the pressure exerted by this pathology has compromised this survival of this species in a few decades since its appearance (McCallum, 2006).

In marine mammals, cancer cases were mostly anecdotal, and prior to the 80's, there were virtually no reports of cases of cancer (Gulland *et al*., 1996). However, some studies showed that occasionally cancer has a high prevalence and can be an important cause of death. For marine mammals, the best-studied case is in the beluga whale, *Delphinapterus leucas*, from the St.

Lawrence estuary, whose population was monitored for 17 years. In this population, cancer prevalence was at around 50%, with an associated mortality of 18%. Most cancers in these animals had origin in the intestine. Cancer was the first adult cause of decease and the second in the entire population (Martineau *et al*., 2002). Between 1982 and 1987 six of 21 belugas had 7 benign tumors, and between 1988 and 1990, 12 od 24 belugas had 21 cancerous tumors. A total of 40% of the population analysed possessed at least one neoplasm and six of the tumors, all of them reported between 1988 and 1990, were malignant. All the animals with cancer had, at least, one adenocarcinoma (de Guise *et al*., 1994).

CSL have been affected by different kinds of cancer including lymphoma, related to gammaherpesvirus OtHV-3 (Ven-Watson *et al*., 2012), lung tumors and others, as presented in Table 1. In general, most types of cancer have a low prevalence in the population. Interestingly, most of the carcinomas appear to originate in the genital tract (Joseph and Cornell, 1986). In 1980, Brown and colleagues (1980) conducted *post-mortem* examinations of female CSL and reported carcinomas that had traits that are consistent with what is now known to be urogenital carcinoma (Gulland *et al*., 1996). Since that original report, the prevalence of urogenital carcinoma in the species has increased dramatically (Lipscomb *et al*., 2010; Browning *et al*., 2015).

Table 2.1. Types of cancer reported for the California sea lion. Modified from Newman and Smith, 2006. ACA=Adenocarcinoma; SCC=Squamous cells carcinoma; TCC=Transition cells carcinoma.

Site	Type of cancer found

Adrenal	Adenoma, Hypernephroma, ACA
Skin	SCC, ACA, Leiomyoma, Fibroma
Uterus	Carcinoma, SCC, Leiomyoma
Ovary	ACA, Adenoma, Granulosa-cell tumor
Tongue	SCC
Lymphatic	Lymphoma
Pancreas	Islet adenoma, Duct adenoma, ACA
Liver	Hepatic carcinoma, Bile-duct ACA
Gingiva	SCC
Lung	SCC, ACA
Pharynx	SCC, Lipoma
Perineum	SCC
Kidney	Fibroma, ACA, Nephroblastoma
Metastatic	Sarcoma, Neuroendocrine
Mammary	Fibrosarcoma, Carcinoma, Infiltrative lipoma, Myosarcoma
Brain	Undifferentiated sarcoma
Vagina	SCC, Papilloma, Hemangioma
Prostate	ACA
Urinary	Papilloma
Bladder	TCC
Pituitary	Adenoma
Eye	Malignant melanoma
Abdominal cavity	Mesenchymoma

2.2 Urogenital carcinoma of the California sea lion (*Zalophus californianus*)

Urogenital carcinoma of CSL has increased in incidence markedly in the past twenty years (Gulland *et al*., 1996; Browning *et al*., 2015). The prevalence of this disease in the population at large is still unknown, but was declared as the cause of death of 18% of animals that stranded along the western coast of California, and underwent post mortem examination (Lipscomb *et al*., 2000). Alarmingly, in 2012 the prevalence of urogenital carcinoma in dead stranded animals reached 26% (Browning *et al*., 2015). In other areas of their distribution, urogenital carcinoma has not yet been reported, although precancerous transformation of the genital epithelium has been detected in CSL from the Gulf of California (Barragán-Vargas *et al*., 2016).

Multiple risk factors are associated with urogenital carcinoma of the CSL. The main actors implied in its development are pathogens, such as a novel otarine gammaherpesvirus (OtHV-1) (Lipscomb *et al*., 2000; Buckles *et al*., 2006) and beta haemolytic streptococci (Johnson *et al*., 2006), high levels of inbreeding (Acevedo-Whitehouse *et al*., 2003a), high concentrations of PCBs and DDT (Ylitalo *et al*., 2005), individual genetic conformation (Bowen *et al*., 2005; Browning and Acevedo-Whitehouse *et al*., 2014) and levels of expression of hormone receptors (Colegrove *et al*., 2009).

The reported associations between urogenital carcinoma and infection by a gammaherpesvirus should not be surprising as related gammaherpesviruses have already been associated with cancer in other wild species before the 80's (Cullen *et al*., 1978; Venn-Watson *et al*., 2012) and also in humans (Taylor and Blackbourn, 2011) and domestic animals (Huang *et al*., 2012). To date, three herpesvirus have been shown to be pathogenic to CSL, namely OtHV-1, OtHV-2 and OtHV3. Of these, only OtHV-1 and OtHV3 have been related to cancer (Lipscomb *et al*., 2000; Buckles *et al*., 2006; Venn-Watson *et al*., 2012).

Gammaherpesviruses typically infect epithelial membranes and leukocytes, commonly entering latency in the latter, especially in B- and T-lymphocytes (Sunil-Chandra *et al*., 1992; Chester *et al*., 1997). These viruses use CD46 as a receptor (Spear and Longnecker, 2003; Cattaneo, 2004) for cell entry, although some bind first to CD21, heparan sulfate, or nectin-1 (the latter, at least, in humans and cattle; Dando *et al*., 2014), which work like anchors to concentrate the virus, helping them to find the receptor essential for fusion (Shukla and Spear, 2001; Spear and Longnecker, 2003). In order to infect lymphocytes, particularly B-cells, some gammaherpesvirus use HLA (MHC-II) as the receptor (Spear and Longnecker, 2003).

Considering that the prevalence of OtHV-1 is highest in adult CSL and that urogenital carcinoma has only been reported in adults, it is likely that the main route of infection is sexual (Buckles *et al*., 2006; 2007). Nevertheless, perinatal transmission between mother and pups has been considered as a secondary route of infection (Buckles *et al*., 2007). Moreover, OtHV-1 has been correlated to urogenital carcinoma in other species (Dagleish *et al*., 2013). However, mechanisms for cancer progression associated to gammaherpesvirus have not yet been described.

Viruses that belong to the Herpesviridae family are characterized by their large size. To colonize the host, they need to evade pattern recognition receptors and they have to modify signalling pathways and cell gene expression (Paludan *et al*., 2011). The virus can avoid the immune system during long-term infections, prior to being to replicate lytically. However, they have a slow replication cycle, prefer latency, and, when active, can be detected by both $CD8^+$ and some innate immune cells (Paludan *et al*., 2011).

Some herpesvirus, such as as Epstein Barr and Kaposi's sarcoma associated-virus, can cause cancer in immunosuppressed individuals (Martin and Gutkind, 2009). Additionally, herpesviruses were related to genital carcinomas in humans in the 60's and 70's, and though to be etiologically

related to cervix cancer, although this was discarded following large-scale studies in the 80's (zur Hausen, 2009). In the CSL, OtHV-1 has been statistically related to urogenital carcinoma but its presence does not determine occurrence of this pathology, and it is likely that other factors are needed for OtHV-1 to have a oncogenic action.

Beta haemolytic streptococci have also been statistically associated with urogenital carcinoma, but only in females (Johnson *et al*., 2006; Colegrove *et al*., 2009). And other studies showed that beta haemolytic streptococci were related to presence of neutrophils and lymphocytes in the genital epithelium of CSL pups within the Gulf of California (Barragán-Vargas, 2013).

Individual genetic predisposition to illness and inbreeding can be related (Keller and Waller, 2002). Not only are individuals with high levels of inbreeding potential pathogen reservoirs; inbreeding can also drive populations towards less efficient immune responses, which can facilitate infections by various pathogens (Acevedo-Whitehouse *et al*., 2003a). Urogenital carcinoma, although not exactly classified as an infectious disease, is correlated with virus and bacterial infections, and sea lions that have lower levels of heterozygosity appear to be more vulnerable to develop this pathology (Acevedo-Whitehouse *et al*., 2003a).

Experiments in animal models have shown that particular alleles in specific genes can influence individual predisposition for cancer. For example, mutations in the *p53* tumor suppressor gene can facilitate cancer progression and spreading (Feng *et al*., 2008). *p53* works correctly if at least one of its alleles produces a correct transcription factor; however, if both alleles are incorrect the probability of tumorous cells appearing increases significantly (Cullen *et al*., 1978). Inbreeding is also relevant in this sense, as consanguineous mating increases the chances of offspring receiving the same allele from both parents, thus increasing homozygosity across the genome and allowing for deleterious recessive alleles to be expressed (Keller and Waller, 2002).

Genetic predisposition to urogenital cancer has been studied in this species in terms of the Major Histocompatibily Complex, MHC, class II DRB genes (termed *Zaca-DRB* in CSL; Bowen *et al*., 2004; 2005) and for the heparanase2 protein (Browning and Acevedo-Whitehouse *et al*., 2014). The MHC is a gene family whose products are expressed as transmembranal proteins on the cell surface of antigen presenting cells. The variable region of these genes is generally very polymorphic. Such high levels of population-level variability are thought to have arisen due to pathogen pressure. In CSL, the MHC class II genes do not present a classical polymorphism with different alleles. Instead of that, each animal possesses different configuration of multiple genes (Bowen *et al*., 2004) and some of these configurations are more common in tbhe population (Bowen *et al*., 2005). The presence of a particular locus, *Zaca-DRB.A*, that was present in 70% of CSL from San Miguel Island (Bowen *et al*., 2006) is related to a higher risk of urogenital carcinoma (Bowen *et al*., 2005). To date, the mechanism that would explain such an association remains unknown.

Individual genetic predisposition to urogenital carcinoma reported to date also includes homozygosity at a particular microsatellite, Pv11, located in the ninth intron of heparanase 2 (Browning and Acevedo-Whitehouse *et al*., 2014). Heparanase 2 has been associated to various carcinomas in humans and, in the CSL, is expressed in animals with urogenital carcinoma which show a homozygous Pv11 allele 1 genotype (Browning and Acevedo-Whitehouse *et al.*, 2014).

Finally, other agents related with carcinoma in CSL are persistent organic pollutants, which can have direct toxicity and be related to carcinogenesis if they are in themselves oncogenic agents or via suppression of onco-immunosurveillance (Beckmen *et al*., 1999; 2003; Ylitalo *et al*., 2005; Acevedo-Whitehouse and Duffus, 2009). Due to the importance that they have for my thesis, I will address them in a separate section ahead.

2.3 Immune responses to cancer

Since the late 20th century, numerous experiments confirmed that cancer cells express specific (altered self) antigens that can be recognized by the immune system in a process known as oncosurveillance. In the 90's, knock-out experiments confirmed that an organism has specific mechanisms for the detection and eradication of cancer (Kim *et al*., 2007). Since then, the main effectors of cancer control and surveillance have been described. Among immune cells involved in oncosurveillance, are B-, T-, NK and NKT cells, and the main oncosurveillance molecules are interferon type I and II, and perforin (Kim *et al*., 2007).

Cancer cells depend on their microenvironment to proliferate and this requires that they establish direct interactions with the surrounding tissues. Such interactions make them detectable by the immune system effectors, so they are under selective pressure to develop immune avoidance traits (Visser *et al*., 2006). Interestingly, the activity of some components of the innate immune system (i.e. macrophages, mast cells, and granulocytes) can contribute significantly to cancer development through mechanisms such as the induction of DNA damage by free radicals, κB nuclear factor dependent intracellular pathways, paracrine regulation, promotion of angiogenesis, and tissue remodelling by synthesis of growth factors, cytokines, chemokines and methaloproteases, overexpression of ciclooxygenase 2 (Visser *et al*., 2006), or suppression of adaptive anti-tumor responses by macrophage polarization (Visser *et al*., 2006; Huang *et al*., 2011); in addition, some adaptive immune responses that typically avoid autoimmunity, such as clonal anergy and decrease of clonal frequency (Malvey *et al*., 1998), can also contribute to tumor development (Visser *et al*., 2006).

The immune system faces tumoral proliferation by three different ways: 1) control of oncogenic viral infection, 2) control of bacterial infections that, in turn, avoids persistent inflammation, and 3) detection and elimination of transformed cells (Vesely *et al*., 2011; Romero, 2011). Detection and control of oncogenic viruses and bacteria can occur prior to transformation or overlap transformation. This phase will be revised later in this section. Because of its relevance to this thesis, oncosurveillance will be described first.

In the elimination phase, tumor detection and recognition, and innate cell mediated elimination start (Kim *et al*., 2007; Vesely *et al*., 2011). Once the tumor acquires a certain size (in humans, 2 to 3 mm), it requires blood support, so it secretes pro-inflammatory molecules. These molecules can also attract and activate some antitumoral cells as NK, NKT and T gamma-delta. These cells recognize the altered self-antigens expressed by the cancer cells, and in response they produce IFN-γ. After that, the anti-tumoral responses increase exponentially. Namely, IFN-γ induces anti-proliferation and pro-apoptosis, and NK cells promote the maturation of dendritic cells (DC), inducing them to migrate to regional lymph nodes in charge of tumor elimination. In addition, NK cells and macrophages both eliminate transformed cells directly (Ruffell *et al*., 2010).

NK cells exert spontaneous cytotoxicity against cells which are deficient in presenting MHC class I. This allows them to respond against transformed cells and against some viral infections (Smyth *et al*., 2005). The NK cytotoxicity can be exerted by two routes; one dependent of tumor necrosis factor and the other dependent of granule exocytosis (Fig. 2.3).

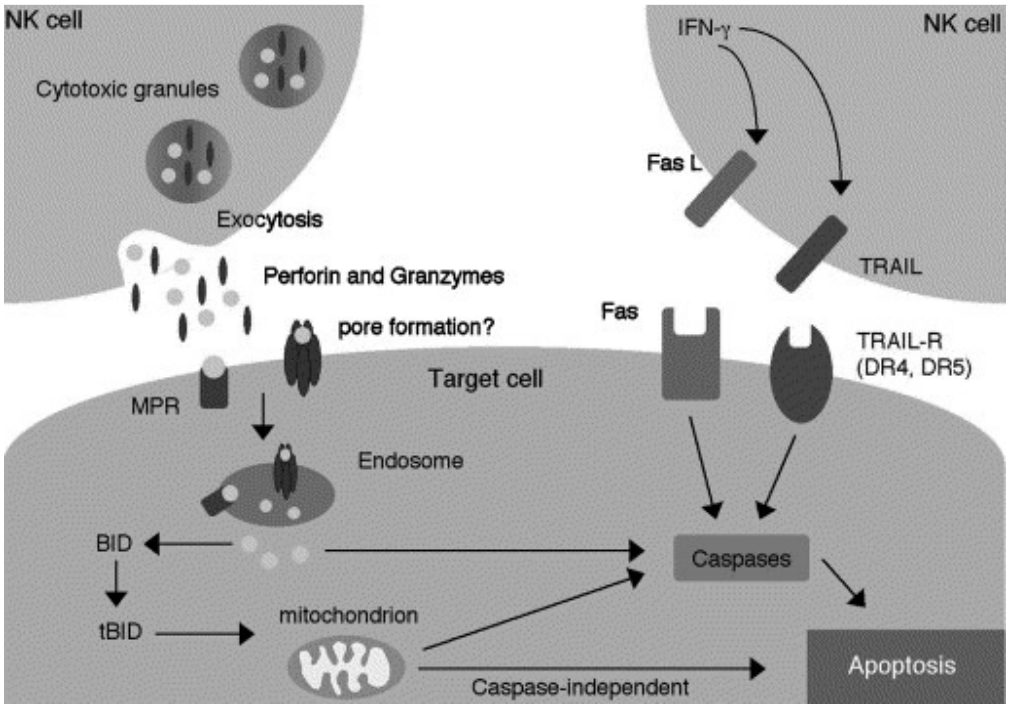

Figure 2.3 Routes of action for NK cytotoxicity (from Smyth *et al.,* 2005).

In the lymph nodes, DCs phagocytose cancer cells and promote the maturation of $CD4^+$ cells. These, in turn, activate tumor antigen specific T cytotoxic lymphocytes. Both main T cell populations, $CD4^+$ and $CD8^+$, infiltrate the tumor and, CTL cells eliminate those cells that express the tumor antigen at their surface (Kim *et al*., 2007; Ruffel *et al*., 2010). Immunorresistant cancer cells, which are those cells that do not express altered antigens and cannot be attacked by T cells, may resist elimination and lead to the next phase termed equilibrium.

During equilibrium, tumors are less immunogenic and are thus less exposed to cytotoxic pressure. Equilibrium occurs mostly in spontaneous tumors, which are not related to known oncogenic factors, since those tumors that were caused by oncogenic virus and those of chemical origin are usually strongly antigenic. The most important effectors during equilibrium are T cells and IFN-γ, and they exert onco-supressive functions, although NK and some support cells can also

maintain their activity. The 'fight' may finally be resolved in favour of the immune cells, and cancer will then be eradicated, but it is also possible that the equilibrium phase is sustained for years or, if cells have limited immunogenicity, the tumor can escape immune surveillance (Kim *et al*., 2007).

When at the escape phase, cancer cells modify their environment through emission of soluble factors or they can incapacitate effector cells via the avoidance of T cell proliferation, inhibition of T cell function or induction of apoptosis of T cells. They can also recruit pro-tumoral macrophages or immature DC that induce an anti-inflammatory response which inhibits cytotoxic T cells (Visser *et al*., 2006; Kim *et al*., 2007; Ruffel *et al*., 2010). Peripheral blood lymphocyte soluble factors, such as FasL, MICA or NKG2 can suppress the activity of cytotoxic cells in different ways, induce apoptosis (Wu and Lanier, 2003; Kim *et al*., 2007), or interfere with the communication between NK and DC (Wu and Lanier, 2003). These effects can be so wide-ranging and strong that immunosuppression can occur in other organs, such as the spleen or the lymph nodes, which in turn favors metastasis (Kim *et al*., 2007).

Although many effectors are implied in the tumor proliferation control, there is a group of cells that are present in all the phases. Lymphocytes, especially NK and cytotoxic T cells, are directly in charge of tumor elimination and, because of that, they are the target of cancer cell counterattack (Rivoltini Licia *et al*., 2002). As there has not yet been a research program that investigates immune responses against cancer for wildlife, taking into account what has been described for humans and laboratory animals, it is parsimonious to focus on these cells as key players of oncosurveillance.

2.2.1 NK cells

NK cells constitute 5-10% of total lymphocytes and around 10-15% of circulating leukocytes. In spite of being lymphocytes, NK cells usually are considered as innate effectors, as they are thought to be unable to respond against a particular antigen (Bryceson *et al*., 2011; Rölle *et al*., 2013) and, until recently, they were considered unable to develop a memory response (Röller *et al*., 2013). Nevertheless, their action birdges between the innate and adaptive responses, and in terms of responses to cancer and viruses, they are main effectors (Bryceson *et al*., 2011; Vivier *et al*., 2011).

To carry out their functions, NK cells have an arsenal that includes the synthesis of IFN-γ and perforins (Bryceson *et al*., 2011; Vivier *et al*., 2011). However, they do not use these molecules except when stimulated by support cells (i.e. macrophages and DC). Depending on the intensity of the stimuli, NK cells can respond by activating LFA-1, expressing MIP-1, degranulating and synthesizing TNFα, or producing IFN-γ (Bryceson *et al*., 2011).

NK cells do not only act through stimuli, they can also recognize unusual cell markers, such as tumor or virus antigens, and the response will depend on their maturation status. In general, low NK activity has been related to increased risk of carcinogenesis (Wu and Lanier, 2003). Lower activity can be product of two mechanisms. First, it can be mediated by less mature or less active cells. NK cell maturation and activation depends on other populations, such as T CD4+ and dendritic cells. The second mechanism relates to a direct decrease in NK cell numbers and delayed maturation or suppression of NK cells that would impact carcinogenesis directly. Also, NK contact with tumor and virus-infected cells will be affected when their circulating numbers are low.

Depending on their maturation status NK cells can belong to different subclasses that vary in terms of their activity. Maturation depends on the level of expression of specific receptors

(Bryceson *et al*., 2011). Immature NK cells ($CD56^{bright}$) are cells that emit regulatory cytokines in response to other cytokines (i.e. interleukins), and they are less cytotoxic than more mature cells (Bryceson *et al*., 2011). Transition NK cells are those that can produce IFN-γ in response to cytokines, and can also produce high levels of perforin which accounts for cytotoxic elimination of abnormal cells. They can also secrete cytokines when recognizing a target cell (Bryceson *et al*., 2011). Finally, mature NK cells produce high levels of cytokines and synthesize the highest levels of perforin, and thus are extremely cytotoxic once they recognize a target cells (Bryceson *et al*., 2011).

NK cells do not only depend on cytokines and presence of target cells for their activation. Their receptors act as intrinsic activation factors (Vivier *et al*., 2008). The response varies depending on which receptors are expressed, and the interaction between these receptors and the target cells (Vivier *et al*., 2008). For example, a cell that has a normal repertoire of transmembrane molecules would not activate any response, regardless of the maturation or activation status of the NK cells. NK receptors can be inhibitory or activating (Wu and Lanier, 2003). Among the NK receptors related to cancer or virus vigilance are Ly49, KIR, NKG (NKG2A and NKG2D), CD244, and NTB-A, which will be described briefly below.

Ly49 and KIR are the best-known NK receptors. Species usually have one or the other, so each receptor has been studied in different models. In the case of the CSL both receptors have been found. Ly49 is a receptor family described in Rodentia (Wu and Lanier, 2003) and Pinnipedia (Parham, 2008; Hammond *et al*., 2009). Ly49 is a membrane receptor that has both activation and inhibitory roles. Its action is based in the recognition of target cells molecules through extracellular lectins, which start an intracellular response cascade. Only one Ly49 gene, with an as yet unknown function, was found in CSL, however, it seems to be similar to the inhibitory proteins found in

rodents. Pinnipeds appear to have only two distinct alleles and these differ in a single nucleotide, which is translated as a non-synonymous substitution in the protein's immunoreceptor tyrosine-based inhibitory motif (ITIM) (Hammond *et al*., 2009). KIR receptors have been described mainly in humans, with high levels of polymorphism (Vivier *et al*., 2008). Its function is to recognize epitopes presented by the MHC. The NK cells eliminate those cells with the epitope (Wu and Lanier, 2003). KIR receptors can also be inhibitory or activators. In general, there is a trend where the longer the intracellular domain length the more likely they are to be inhibitors, while shorter domains tend to be activators. The KIR gene was recently described in pinnipeds. Although its role has not been confirmed yet for pinnipeds, it appears to produce functional membrane proteins (Hammond *et al*., 2009). The only gene described for this receptor in CSL presents low polymorphism (Parham, 2008; Hammond *et al*., 2009). The protein presents two extramembrane domains, as does the human's KIR, bit in other pinnipeds there are three protein domains (Parham, 2008; Hammond *et al*., 2009).

The NKG family has not been described yet for pinnipeds but, according to the NCBI database, its sequence has been predicted for the Wedell seal, *Leptonychotes wedellii*, and the walrus, *Odobenus rosmarus*, as well as for a closely-related terrestrial carnivore, the domestic dog, *Canis lupus familiaris*. Thus, its existence in the CSL is highly likely. NKG2 works as an activator protein in NK. It links to many cell ligands as retinoic acid 1 (RAE-1), MULT-1 and the minor histocompatibility antigen H60. RAE-1 is overexpressed in most of tumoral cells and is also induced by viral infection, so NK cell activation is responding to cancer actual processes or potential processes NKG2A activates the same signalling pathway as KIR inhibitory proteins and inhibits NK cytotoxic activity (Wu and Lanier, 2003; Lanier, 2008) and responds to total MHC expression reported by HLA-E in an unknown way (Wu and Lanier, 2003). NKG2D produces such

a strong activation of NK cells that activity can overcome MHC-mediated inhibition (Wu and Lanier, 2003).

ITAM dependent signalers are a heterogeneous membrane receptor group that activates cytotoxic response in NK cells. They include CD16 and natural cytotoxicity receptors (NKp30, NKp44 and NKp46). These receptor complexes are NK activators involved in the elimination of tumor cells, although their related tumor ligands are still unknown (Wu and Lanier, 2003; Tsukerman *et al*., 2014). CD16 activates NK cells via an interaction with IgG (Tsukerman *et al*., 2014). To date, most of its other ligands remain unexplored (Wu and Lanier, 2003).

CD244 and NTB-A are two CD2 family receptors that activate cytotoxicity and modulate IFN-γ secretion (Flaig *et al*., 2004). CD244 cooperates with ITAM bearing receptors and it increases integrin dependent NK activation (Wu and Lanier, 2003). In humans, it can recognize other members of the same family, particularly CD48 (Flaig *et al*., 2004). NTB-A is found in NK, T and B cells and orchestrates immune response through cross-communication. It needs other NK receptors, as NKp30, NKp44 and NKp46, in order to induce cytotoxicity (Flaig *et al*., 2004).

Activation or inhibition of NK cells is dependent on the balance among these receptors. Since somatic cells can become transformed by various mechanisms including infections by oncogenic viruses and carcinogenic chemicals, an adequate number of NK cells is essential for oncovigilance and control of cancer (Wu and Lanier, 2003).

2.2.2 Lymphocytes

NK cells are not the only population implicated in oncosurveillance. Other lymphocytes also have a main role in the development or suppression of cancer (Beyer and Schultz, 2006; Ruffel et al., 2010). The tumor microenvironment attracts immunosuppresor myeloid cells. These cells, however, can recruit lymphocytes with antitumorogenic or protumorogenic activity. Depending on the infiltration and class of these lymphocytes, diagnosis can determine if a tumor can develop or if it will be inhibited (Ruffel *et al*., 2010). Lymphocytes conform the adaptive immune system and are classified as B- cells and T cells, that encompass $CD8^{+}$, $CD4^{+}$ helper, and Treg ($CD4^{+}CD25^{high}FoxP3^{+}$) cells. All of them have different actions and roles.

B cells are responsible for the adaptive humoral responses. These cells are characterized by having BCR receptors, also known as antibodies, which can be attached to the cell membrane or released in the blood stream. It is not yet clear whether circulating B lymphocytes have a direct role in cancer control although an indirect role has been described. Namely, B cells regulate the activities of other lymphoid subpopulations and help control or erradicate extracellular pathogens, thus limiting chronic inflammation that could have otherwise lead to tumorigenesis. However, two B-cell subpopulations can influence cancer development. B regulators (Breg) can exert a pro-tumoral activity while they protect the body from autoimmune reactions. Breg cells attract Treg cells towards the tumor and as this population produces antiinflamatory cytokines, the result can be pro-tumoral (Beyer and Schultz, 2006; Mauri and Bosma, 2012). For example, it has been proposed that the elimination of this lymphocyte subpopulation could be an antitumoral therapy (Inoue *et al*., 2007). On the other hand, B cells that infiltrate tumors (usually named TiBc) are associated with a positive prognosis. Although their mechanism of action remains unknown, it has been reported that they can substitute antigen-presenting DC in lymph nodes (Linnebacher and Matletzki, 2012).

T lymphocytes possess a T receptor (TCR) that allows them to recognize antigens presented by the MHC. Cytotoxic T cells ($CD8^+$) recognize those presented by MHC class I whereas $CD4^+$ recognize those presented by MHC class II expressed on antigen-presenting cells (APC; i.e. monocytes and DC) (Ruffel *et al*., 2010). T $CD8^+$ cells were the first population related to tumor control to be described. Together with NK cells they seem to be the main mediators of antitumoral responses, as they produce IFN-γ and thus directly eliminate affected cells. Tγδ cells share this mechanism, but they only operate in epithelial tissues (Ruffell *et al*., 2010).

$CD8^+$ cells protect against primary infections by viruses and also protect against viral reactivation if these had not been inactivated. In addition, they recognize tumor antigens, which means that they exert a strong response when a cell faces both kinds of damage (Ruffell *et al*., 2010). Tumors associated to oncogenic viruses, like Herpesvirus-8, increase if T cells do not function correctly. Some tumors can avoid $CD8^+$ cell detection through direct or indirect inhibition (Ruffell *et al*., 2010).

NK and T cells are mediated by cytokines and altered-self antigens presented by neoplastic cells, stromatic cells or other leukocytes. T $CD8^+$cells require IFN-γ (secreted by NK cells) to mature and then can promote further NK action. Such regulation, necessary to avoid an autoimmune response, can be exploited by tumorous cells in order to avoid antitumoral responses (Ruffell *et al*., 2010). This can work either by decreasing the $CD8^+$ population or diminishing their activity level. The $CD8^+$ activity can be determined using the EOMES transcription factor as marker (Pearce *et al*., 2003).

2.2.3 Support cells

In addition to NK and cytotoxic T cells, there are other cells, such as those that express $CD3^+$ T cell co-receptor, that are implied in tumor control via regulation of the cytotoxic lymphocyte population. The most important regulators among $CD3^+$ cells are T $CD4^+$ and Treg.

T $CD4^+$ cells are in charge of cellular regulation through the emission of activation and inhibitory cytokines. Depending on the organ these cells are in, their action can stimulate or inhibit carcinogenesis. For example, in mice, T $CD4^+$ cells found in cervical tumors increase the tumor intensity whereas in the skin they reduce tumor growth (DeNardo *et al*., 2010). Whether the effects of T $CD4^+$ will be pro- or anti-tumor will depend on which kind of response is driven by these cells, similarly to what happens in macrophages in which M1 works against infections and M2 drives a immunotolerant response. (Aras and Zaidi, 2017). Th1 responses are associated with a good prognosis, as these Th1 $CD4^+$ cells recruit T $CD8^+$ and NK cells, whereas Th2 $CD4^+$ exert anti-inflammatory response with a tumoral progression prognosis. Additionally, $CD4^+$ cells can elicit other responses; among them, modulatory responses driven by regulatory T cells (Tregs). This kind of response is tumor-dependent and, thus, can have both anti- or pro-tumoral activities (Ruffell *et al*., 2010).

Proinflammatory response is associated with anti tumoral activity, and it is named Th1. $CD4^+$ cells exerting Th1 response secrete IFN-γ, TNF-α (DeNardo *et al*., 2010; Ruffel *et al*., 2010), IL2, and IL12. Moreover, they increase antigen presentation capacity of APCs, they increase the magnitude of $CD8^+$ responses, and they even can kill tumor cells overproducing IFN-γ, TNF-α and synthesis of cytolytic granules (De Nardo *et al*., 2010). $CD4^+$ cells express the transcription factors STAT1, STAT4 (Zheng and Favell, 1997), and Tbet (Dorfman *et al*., 2003) and, thus, they can be characterized with them.

Antiinflammatory Th2 responses can be associated to development of cancer. These cells secrete IL4, IL5 (DeNardo *et al*., 2010; Ruffel *et al*., 2010), IL6, IL10 (DeNardo *et al*., 2010), and IL13 (DeNardo *et al*., 2010; Ruffel *et al*., 2010) that reduce $CD8^+$ cytotoxicity and promote the B-dependent humoral response. Some of their interleukins have been linked to determined tumors. For instance, high levels of IL4 are related to mammary cancer (Aspord *et al*., 2007). In addition, a higher Th2:Th1 ratio is related to a bad prognosis of cancer (DeNardo *et al*., 2010). $CD4^+$ showing a Th2 profile, typically express transcription factors such as GATA3, STAT6, NF-IL6, NF-AT and AP-1 that can be used to determine a Th2 profile (Zheng and Favell, 1997).

Th17 is not specifically related to anti- or pro-inflammatory responses; rather, its action will depend on the tissular environment (DeNardo *et al*., 2010). Th17 cells are characterized by a high expression of IL17, which stimulates angiogenesis and vascularization so its presence tends to be associated with a bad prognosis (DeNardo *et al*., 2010, Wilke *et al*., 2011). However, as was said before, the presence of IL17 can also be related to antitumoral actions, as in anti-metastasis protection (DeNardo *et al*., 2010). Its action is dual and depends on their localization (DeNardo *et al*., 2010).

As Th17 is produced in low quantities, its presence is not a good index for inferring numbers of Th17 cells. Circulating Th17 $CD4^+$ cell counts usually do not provide information since peripheral blood levels are similar in healthy and cancerous individual (Wilke *et al*., 2011). This does not mean that the Th17 cells populations are constant, as they increase near the tumor, and this higher presence is related to recruitment of cytotoxic T and NK cells, (Wilke *et al*., 2011).

T regulator (Treg) cells, which express the transcription factor FoxP3 (Hori *et al*., 2003; Beyer and Schultz, 2006), are gaining attention for their role in cancer as our understanding about them increases. Their main function seems to be suppression of anti-tumoral activity as a mechanism to

avoid autoimmune reactions. They exert this action in cell-to-cell contact in a dose-dependent way. They can inhibit $CD4^+$; $CD8^+$, driving to a suppression of long-term cytotoxic activity; and also they can impede DC, NK, B and NKT cells action, suppressing the innate cytotoxic activity (Beyer and Schultz, 2006, Ruffell *et al*., 2010). This works in both ways, as Treg cells are not the only cells that stop the effects of other cells. Treg cells are regulated by DC, which can drive Treg into Th17 cells through IL6 action, thus allowing for increasing numbers of $CD8^+$ cells near the tumor (Wilke *et al*., 2011).

In addition to their role in cancer, Treg and Th17 cells have an opposite role in autoimmune processes. A high number of Th17 cells can favor carcinogenesis although it remains unknown whether, *in vivo*, the increase of these cells is a cause or a consequence of tumor progression (Beyer and Schultz, 2006). An increase of circulating Treg cells is observed in some cancers. For instance, an increase of Treg in prostate dysplasia in mice is correlated with tumor progression (Beyer and Schultz, 2006).

2.3 Persistent organic pollutants in the environment

Persistent organic pollutants (POP) are chemicals that remain in the environment for long periods of time. POP are generated by industrial or agricultural processes, such as the use of pesticides, which despite their current prohibition in many countries, were widely disseminated during the last century, especially during the 1960's and 70's (Gabrielsen, 2007; Ludewig *et al*., 2013). These chemicals are ubiquitous, they are found in the environment, within the bodies of organisms, and, thus, along the food chain, and they can exert a toxic effect on their own or combined with other chemicals (Beckmen *et al*., 2003; Ludewig *et al*., 2013). POPs are stable and resistant to

degradation due to the presence of halogens such as iodines or bromines (Ludewig *et al*., 2013). Especially important among POP are organochlorines (polychlorinated biphenyls, PCB; and di-chloro di-phenyl trichloroethane, DDT), as they are widely distributed and are related to a wide spectrum of health issues. In general, high doses drive acute toxic reactions in animals while lower dosis tend to lead to immunosuppresion (Beckmen *et al.*, 2003).

The association between POP and cancer has been studied for a long time under laboratory conditions (Preston *et al*., 1981; Kimbrough *et al*., 1975). Mainly, carcinogenicity of POP has mostly been related to liver cancer in rats and mice, although tumors in other tissues have also been reported. Carcinogenicity can be directly induced by due some of these pollutants, with effects that include direct genotoxicity, promoting tumor cells and leading to tumor progression (Ludewig et al., 2013). Not all POP act equally. Some act as promotors or initiators, while others are direct carcinogens (Ludewig *et al*., 2013). However, although pollutants are usually found as combinations, rather than on their own, in the envionment (Pacheco, 2013), effects can be difficult to predict.

POPs can influence carcinogenesis indirectly by leading to the production of Reactive Oxigen Species (ROS) that can exert complete carcinogenesis, disrupting DNA or proteins in the cell; or they can interfere metabolic pathways, which can destabilize organism and, thus, impede the normal homeostasis and cell proliferation control (Ludewig *et al*., 2013). Notwithstanding, one of the most frequently reported effects of environmental pollutants is immunosuppression, which can lead to cancer (Beckmen *et al*., 1999; 2003; Ylitalo *et al*., 2005; Acevedo-Whitehouse and Duffus, 2009). Immunosuppression can facilitate not only the tumorous cell evasion of the immune system, but it can also increase the risk of infection and the persistence of oncogenic pathogens (Lecuit and Eloit, 2013).

The presence of POP in the marine environment has been shown to affect the dynamics of pinniped populations. For example, the Steller sea lion (*Eumetopias jubatus*) population began to decline in the 70's. For twenty years, nutritional stress and competion with fisheries were thought to be the main causes of the decline. However, since the 90's, evidence of a direct association between pollutants and population decrease was reported (Barron *et al*., 2003; Atkinson *et al*., 2008). Due to their thick blubber layer, their position in the trophic web, and their longevity, the Steller sea lion and other large marine mammals tend to acquire high concentrations of aromatic and hydrophobic compounds (Beckmen *et al*., 1999; Ylitalo *et al*., 2005; Blasius and Goodmanlowe, 2008; McAloose and Newton, 2009; Bossart, 2010) which would expectedly favor the intensity and development of carcinogenicity. There is some evidence of this, as correlations between carcinogenesis and the presence of POP have been reported for the CSL (Ylitalo *et al*., 2005; Blasius and Goodmanlowe, 2008). These relationships could be related to the loss of blubber during severe illness, caquexia, or during long fasts. Under these circumstances, organochlorines accumulated in the fat are mobilized and they can have a toxic impact (Ylitalo *et al*., 2005).

The harming effects of OC would particularly affect young animals due to their lipophylic traits that allow them to be transfered in maternal milk. Lactating pups acquire OC through milk that has high lipid content, and they accumulate in their blubber. The levels of these pollutants aer related to poor survival in northern fur seal, *Callorhinus ursinus*, pups. The first-born pup acquires greater amounts of OC due to maternal offloading of the pollutants that had accumulated in the blubber throughout her life (Beckmen *et al*., 1999; 2003). In addition, transplacentary offload leads to pups having up to six times the contaminant levels of their mothers (Beckmen *et al*., 1999). Female lypophilic pollutant concentration decreases with age, due to nursing and birth (Meng *et*

al., 2010). In pups, such high levels of OC can affect the developing immune system (Beckmen *et al*., 2003).

DDTs are transferred to the pups via nursing more easily than PCBs, as they are more soluble and there is a selective barrier dependent on their solubility that favors them with respect to less chlorinated organochlorines (Beckmen *et al*., 1999). Because of this, DDT accumulation starts before PCB accumulation. The later accumulate mainly through the diet. Adult male CSL tend to have lower PCB levels because they feed in less-polluted sites (Ylitalo *et al*., 2005) but throughout their life the consumption of polluted prey usually leads to their bioaccumulation (Meng *et al*., 2010).

There are many factors to consider before determining a casual mechanism in ecological studies. However, some research supports lymphoproliferative responses are negatively related to organochlorines in blood (Beckmen *et al*., 2003). Laboratory experiments conducted on the common marmoset (*Callithrix jacchus*) show that lymphocyte subpopulations vary between chronically dioxin-treated individuals and control animals (Neubert *et al*., 1992). Such findings constitute evidence that pollutants can modify the immune responses of an individual. Previous reports shown that immune cell subpopulations vary depending on exposure to specific pollutants (Neubert *et al*., 1992).

2.3.1 Routes of action of organochlorines

According to the International Agency for Research on Cancer (IARC), POP are carcinogenic, by direct and indirect mechanisms. The IARC classifies PCBs in two different categories. Category

1 (which includes PCB126) and 2A (which includes most of the other PCB congeners) correspond to molecules considered carcinogenic or probably carcinogenic to humans, respectively (IARC, 2009). DDT is classified as 2B (IARC, 1991).

Organochlorines have multiple effects at different levels. At the molecular level, their structure allows them to attach to cytosolic receptors, such as the aryl hydrocarbon receptor (AhR), which modulates the immune response (Wolff *et al.*, 1993) through differentiation between Treg and Th17 cells (Quintana *et al*., 2008). In addition, DDT and some PCBs are estrogen-like molecules (Roy *et al.,* 2009) and can cause endocrine disruptions that lead to breast cancer (Wolff *et al*., 1993) and other hormonal issues (Roy *et al.,* 2009). Organochlorines are also associated to mutation, as they increase the amount of ROS, and can be directly genotoxic (Sandaj *et al.*, 2008). Most of their actions are directly or indirectly related to destabilization of the cell cycle, for example, through the NFkB transcription factor (Glauert *et al.*, 2008), so they can induce cancer and can also facilitate its progression.

These molecular effects depend on the configuration of the organochlorine. There are different categories of PCBs, such as *para* and *meta* PCBs (Robertson and Hansen, 2001) and mono-*ortho* (MO), di-ortho (DO) and no-*ortho* (NO) PCBs (Levin *et al.*, 2005). Some MO congeners (i.e. 105, 114, 118, 123, 138, 156, 157, 167 and 189), NO (77, 126 and 169) PCBs have a dioxin-like action through AhR (Roberston and Hansen, 2001; Levin *et al.,* 2005), as do chlorinated dioxins (PCDD) and chlorinated furanes, PCDF (Levin *et al.,* 2005). DO congeners exert action via AhR-independent mechanisms (Robertson and Hansen, 2001). DDT mechanisms of action are similar to MO and NO, and they act as inhibitor of P450, an enzyme activated by AhR (Robertson and Hansen 2001), although their toxic effects tend to be lower in mammals than in other animals (Wojtowicz *et al.,* 2011).

Dioxin-like action is the most studied effect of PCBs. For a long time, commercial mixtures of PCBs, such as Aroclor 1254, have been tested to determine its toxicity and effects were attributed to its dioxin like compounds. This mixture produces thymic atrophy in mammals and decreases in lymphocyte population in birds (Andersson *et al.* 1991). The relevance of thymic atrophy has been shown with athymic nu/nu mice, which are the usual model to test teratogenic capacity of transformed cells, since they lack functional T and NK cells and are thus prone to oncogenesis (Zhang *et al.*, 2012). Moreover, oral exposure to Aroclor 1254 diminishes NK cell activity in rats (Exon *et al.*, 1985; Smialowicz *et al.*, 1989).

Dioxin action works through AhR and androstane hormone receptors, which responds to cyclic molecules to induce or inhibit production of P450 (Robertson and Hansen, 2001; Levin *et al.*, 2005). P450 catabolyzes hormones, such as testosterone and estrogens, and OCs (Robertson and Hansen, 2001).

Catabolism of highly chlorinated OCs is slower and can be toxic to the organism as the intermediate metabolites can be as toxic, or more, than the original substrate (Robertson and Hansen, 2001). Intermediate metabolites can have toxic or biochemical effects, depending on the parent compound.

Low chlorinated PCBs are hydroxylated *in vivo* and *in vitro* by P450 isoforms, which can lead to their activation (Safe, 1989). Arene oxide is one of the by-products of PCB metabolism (Safe, 1989; Mc Lean *et al.*, 1996) and act as a strong electrophile that can attack cells.

PCBs can be oxidized to semiquinone by enzymes such as peroxidases, prostaglandine synthase or P450, and produce electrophilic molecules. Arene oxydes and semiquinones can react with nucleophilic compounds in the cell (Amaro *et al.* 1996) as well as with nucleotides, especially with purines, and form adducts (Mc Lean *et al.*, 1996). Adducts may interfere with the proper

function of DNA (Jeong *et al.*, 2008; Spencer *et al.*, 2009) and drive the cell to transformation. Low chlorinated PCBs can produce ROS and, consequently, oxidative stress (Oakley *et al.*, 1996, Srinivasan *et al.*, 2001). Oxidative stress produces 8-oxodeoxyguanosina, a non-natural base that transforms guanine to thymine. In addition, hydroxil radicals can attack lipids and produce metabolites that modify DNA bases, forming adducts with deoxyadenine, deoxyguanine and deoxycitosine. These unions foster substitutions and deletions and, consequently, produce mutagenicity (Srinivasan *et al.*, 2001).

The mechanisms of action of non-dioxin like compounds (DO) are less understood as these molecules were long considered to be biologically inactive although they are more represented in nature due to biotransformation and bioaccumulation (Fischer *et al.*, 1998). However, recent studies have shown that they exert diverse effects *in vivo* and *in vitro* in mice, humans and marine mammals (Fischer *et al.*, 1998; Levin *et al.,* 2004; Levin *et al*., 2007; Mori *et al*., 2008).

Among the reported molecular effects there are *in vitro* alterations in calcium homeostasis, with opening of channels that increase intracellular Ca^{2+} (Fischer and Wagner, 1998; Ganey *et al*., 1998; Kodavanti, 1998; Pessah and Wong, 1998). They can also initiate production of O_2^- in neutrophils (Ganey *et al*., 1998). These effects seem dependent on the chlorine position in addition to its coplanarity (Fischer *et al.*, 1998). Also, as non-dioxin like PCBs have weak unions to AhR, they could exert a similar behaviour to that of dioxin-like PCBs but with lower effects (Seegal *et al.*, 1998).

As a consequence of these actions, dioxin like PCBs effects on individual include weight loss, thymic atrophy, immunosupression, porphyria, and hepatotoxicity (Safe, 1984; Robertson and Hansen, 2001). Non-dioxin like compounds, and high concentrations of dioxin like, produce other toxic effects such as direct carginogenesis, neurotoxicity, and behavioural and endocrine changes

(Safe, 1984; Robertson and Hansen, 2001), which include irritability, muscular incapacity, incoordination, and deficit of cognitive capacity (Safe, 1984; Royland and Kodavanti, 2008). Highly chlorinated PCBs can cause neoplastic injuries in the liver of exposed animals (Safe, 1984). Lower chlorinated PCBs can produce pre-neoplasic injuries without tumor formation and can also prolongate the animal life, probably, by enhancing the immune system (Safe, 1984, Shin *et al.*, 2013).

In studies conducted *in vitro*, PCB effects include higher reactivity of the cell, increased or decreased cytotoxicity and variations in lymphocyte proliferation (Lahvis *et al.*, 1995; de Guise *et al.*, 1998; Levin *et al.*, 2005; Mori *et al.*, 2008). It has been shown that organochlorines exert direct mutagenicity on lymphocytes (Sandaj *et al.*, 2008) or on their precursors in the bone marrow (Meisner *et al.*, 1992), so immune responses can decrease due to the accumulation of genetic aberrations. These effects can lead to leukemia (Ward *et al.*, 2009) or autoimmune diseases, although organochlorine effects on thymus reduction is a more plausible explanation for the latter (Gogal and Holladay, 2008).

In addition to effects on lymphocytes, epithelial proliferation can be affected by organochlorine and intermediate metabolites of organochlorine degradation. As mentioned before, uncontrolled proliferation is necessary for the progression of carcinoma (Hanahan and Weinberg, 2000; 2011) and it is associated to estrogen and progesterone receptors (Cunha *et al.*, 2004), which have already been related to urogenital carcinoma in CSL (Colegrove *et al.*, 2009). Thus, even if organochlorines would not affect the immune system directly, they can help to promote carcinogenesis and endocrine problems (Roy *et al.,* 2009).

2.4 The California sea lion (*Zalophus californianus*) as a study model

Pinnipeds can be considered sentinels of the marine ecosystem as they possess a series of characteristics that can reflect the status of the marine environment. They are long-lived top predators that live near the coast for extended periods of time, and they bioaccumulate anthropogenic toxins in their blubber (Beckmen *et al*., 2003). Furthermore, as they generate an emotional impact in humans, they can be useful to focus social consciousness in environmental problems (Bossart, 2010).

The CSL is the animal model of this thesis. The species has an external ear flap, two pairs of mammary glands, short fur, and a thick blubber layer (Berta, 2009). The blubber makes them vulnerable to lipophilic organic pollutants, especially during fasting, as these contaminants accumulate in the fat. The species belongs to the Otariidea family, within the superfamily Pinnipedia (Berta, 2009) and inhabits from the central Mexican Pacific to British Columbia, in Canada (NMFS, 2013). The current study focuses on CSL along their distribution in the Gulf of California, the Baja California Peninsula and the California coastline.

In Mexico, the CSL is a protected species under the *Norma Oficial Mexicana* (NOM-0959-ECOL-2001) and all of its colonies are located within protected areas (Labrada, 2003). Since 1972 the CSL was included in the US Marine Mammal Protection Act (MMPA, 1972). Such protection status suggests that the only real danger to its populations is habitat degradation (Aurioles, 1993) but there are other potential threats such as pesticide pollution, uncontrolled fishing, introduction of exotic species, eco-tourism and climate change (Zavala-González *et al*., 2004). The species' habitat is affected by natural environmental fluctuations, such as ENSO (El Niño Southern Oscillation). This periodical event generates stress and starvation in CSL populations for long

periods. Other climatic alterations can also affect the population. For instance, between 2013 and 2015 a mass of warm water was detected in northeastern Pacific (termed 'the Blob') lead to unusal mortality events of CSL (Kintisch, 2015). Environmental conditions that decrease prey availability will mobilize blubber stores and, thus, will increase blood POP concentrations. Plausibly, severe climatic anomalies could modify the incidence of urogenital carcinoma.

The CSL is currently acknowledged as a model in which to study tumor progression in (Browning *et al*., 2015) and immune responses have been studied from different perspectives so there is ample literature about these topics. The species is relatively easy to capture and sample as it has an amphibious life and reproduction and nurturing are carried out in land. The male establishes its territory in a beach or on rocks. In these territories, females arrive to give birth to their pups and to mate again. Normally, adult females give birth to one pup each year and they nurse them during their first year of life. This allows the capture of pups and females on the breeding colonies.

2.4.1 The California sea lion population

Around 10% of the total CSL population is found in Mexican waters, whereas the rest of population, estimated as approximately 380,000 individuals (Aurioles-Gamboa and Hernández-Camacho, 2015) lives along the US and Canada coastlines. According to genetic studies, the CSL population has been divided in two big groups, namely the Gulf of California and the northeastern Pacific (Maldonado *et al*., 1995).

Within the Gulf of California, ecological regionalization studies have divided the population according to demographic trends and variations in the region I of the mtDNA control region. Demographic trends defined four regions (Szteren *et al*., 2006), namely the northern region (harboring colonies Rocas Consag, Lobos, and San Jorge), the north-central region (with colonies Granito, Cantiles, and Los Machos), the south-central region (with colonies Partido, Rasito, San Esteban, San Pedro Mártir, and San Pedro Nolasco) and the far south region (Los Islotes and Farallón de San Ignacio). Molecular variation at the mtDNA control region I support such regionalization (Schramm *et al*., 2009), by defining three regions: northern (Lobos, Granito and San Jorge), central (San Esteban and Cantiles) and southern (Los Islotes).

CSL from US waters are presumably affected by highly industrialized coastal areas, pollutants accumulated in marine environments, and CSL from the Pacific coast of the US show PCB and DDT concentrations that exceed the threshold reported for health (Blasius and Goodmanlowe, 2008). In contrast, CSL populations from Baja California and the Gulf of California inhabit relatively less industrialized areas, where organochlorine pollution is lower (Szteren and Aurioles, 2006; Niño-Torres *et al*., 2009) (see Table 2.2).

Table 2.2. DDT and PCB blubber concentrations in CSL sampled in the California coast and in the Gulf of California.

	California (Ylitalo *et al.,* 2005)		Baja California (del Toro *et al.,* 2006)		Golfo de California (Niño-Torres *et al.,* 2009)	
Pollutant	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation

DDT in lipids	380.000 ng g-1 (males) 250.000 ng g-1 (females)	480.000 ng g-1 (males) 440.000 ng g-1 (females)	3.780 ng g-1	Not reported Rank 1.880-7.870	3.400 ng g-1 (males) 2.100 ng g-1 (females) 1.400 ng g-1 (pups)	1200 ng g-1 900 ng g-1 380 ng g-1
PCB in lipids	77.000 ng g-1 (males) 83.000 ng g-1 (females)	79.000 ng g-1 (males) 160.000 ng g-1 (females)	2.960 ng g-1	Not reported Rank 1.200-9.500 ng g-1	1.600 ng g-1 (males) 1.300 ng g-1 (females) 1.800 ng g-1 (pups)	700 ng g-1 400 ng g-1 g-1

3. STUDY JUSTIFICATION

Urogenital carcinoma has a high prevalence among CSLs in California, with 26% of stranded animals that underwent a *post-mortem* examination showing evidence of the disease (Browning *et al*., 2016). This pathology has a multifactorial etiology that includes infection by OtHV-1 (King *et al*., 2002; Buckles *et al*., 2006), exposure to contaminants (Ylitalo *et al.,* 2005), and genetic factors (Acevedo-Whitehouse *et al.,* 2003a; Bowen *et al.,* 2005, Browning and Acevedo-Whitehouse *et al.*, 2014). In a simple model of carcinogenesis, OtHV-1 infection could be the first step, necessary but insufficient by itself as an explanation for urogenital carcinoma, as the virus is found in CSL from Mexican waters, and was not found to be associated to pre-cancerous transformation (Barragán-Vargas *et al.*, 2016). So, it is likely that a second event is needed. If we were to accept that the Pacific Ocean population is relatively homogeneous from Southern California to Baja California (Maldonado *et al*., 1995; Schramm *et al*., 2009), where carcinoma has not yet been reported, the second event could plausibly be of environmental origin, as there are marked differences in the levels of OC between CSL from the California coast and CSL from the Mexican Pacific and Gulf of California (Ylitalo *et al*., 2005; Del Toro *et al*., 2006; Niño-Torres *et al*., 2009). CSL populations along the California coast have higher OC concentrations, which probably drive this population to be more susceptible to illness, particularly urogenital carcinoma. In other marine mammals, an immunosuppression threshold for OC has been confirmed (Jepson *et al*., 2005). Above this threshold, the immune system cannot eliminate infections correctly, so a higher parasite burden and associated diseases commonly occur. Although any type of immune suppression will affect an individual's ability to fight infection, lymphoid populations are especially sensitive to OC-mediated immunosuppression, which can even atrophy the thymus if

exposure occurs during early development. Lymphoid populations such as T and NK cells are in charge of anti-viral and anti-tumorogenic responses (Vivier *et al*., 2008; 2011). OC-driven immunomodulation of these cell populations is a plausible cause of urogenital carcinomas in the CSL, and this thesis aims to examine this possibility and provide valuable information about the effects of environmental pollution on key immune effectors for viral- and oncovigillance.

In addition, urogenital carcinoma is one of the few types of cancer described in non-domestic animals (Browning and Acevedo-Whitehouse *et al*., 2014) and the CSL is a suitable model to understand how this kind of pathology develops under natural conditions. To date, studies on urogenital carcinoma have been mainly performed in stranded animals, which can bias the results. This work will focus in both stranded and captured free-ranging animals to attempt to find the immune mechanisms related to cell transformation.

The research question that gave rise to this PhD dissertation was focused on discerning the role of the organochlorines PCB and DDT on the CSL immune system, particularly, on the cells that oversee oncogenesis and viral infections, and also on examining how the CSL immune responses vary among different areas of their distribution.

4. VALIDATION OF *IN VITRO* ASSAYS TO DETECT NK CELL-LIKE CYTOTOXICITY

4.1. Introduction

The *in vitro* measurement of natural killer (NK) cell activity has been widely used as an indicator of innate cytotoxicity, antitumorigenic ability and prognosis of metastasis in humans and laboratory animal models. This assay has been the basis of various studies (Anfossi *et al.,* 2015), including those focused on health status (Kusaka *et al.,* 1992) and viral diseases (Almerigogna *et al.,* 2011). In humans, mice and other animal models (Knapp *et al.,* 1993; Mata *et al.,* 2014). NK cell activity is generally measured in samples of circulating blood cells (Bryceson, 2006; Tarazona *et al.,* 2015) using flow cytometry (Somanchi *et al.,* 2015; Tarazona *et al.,* 2015) or radioactive Cr51 (de Guise *et al.,* 1997; Somanchi *et al.,* 2015). However, despite its usefulness, the intrinsic difficulties of procuring blood samples from wildlife and being able to process them immediately after collection, limit the wider application of this immune assay for free-ranging wildlife. The relatively few studies that have been carried out in wild vertebrates, such as common roach fish (*Rutilus rutilus*; (Salo *et al.,* 2000), loggerhead turtles (*Caretta caretta*; (Rousselet *et al.,* 2013), Caspian turtles (*Mauremis caspica*; (Muñoz *et al.,* 2000), beluga whales (*Delphinapterus leucas*; de Guise *et al.,* 1997), harbor seals (*Phoca vitulina*; (Ross *et al.,* 1996) and grey seals (*Halichoerus grypus*; (Hammond *et al.,* 2005) have usually been performed on fresh blood samples collected from animals maintained in managed care facilities (Ross *et al.,* 1996; de Guise *et al.,* 1997) or from free-living individuals captured at sites that are near to the processing laboratory (de Guise *et al.,* 1997), as these fresh samples will only remain viable for a limited time after collection.

When studying wildlife, fresh carcasses constitute an important source of biological samples (Morton and Perrin, 1997). This is the case for marine mammals, where stranded animals represent a valuable source of samples and information related to genetics, microbiology, pathology and toxicology (Borrell and Aguilar, 1990; Cameron *et al.,* 2008; Duignan, 2003; Foote *et al.,* 2009; Luksenburg *et al.,* 2015; Uhart *et al.,* 2009). However, collection of blood samples from carcasses is difficult, as blood coagulates quickly after death, and sample volumes tend to be insufficient to extract peripheral blood mononuclear cells (PBMC) for immune assays. On the other hand, lymph nodes contain high numbers of mononuclear cells (LNMC), and the tissue remains viable for culture up to 24 hours after the animal's death (Pugliares *et al.,* 2007). Similar to PBMC, LNMC can be useful to monitor individual's immune functions and health status, and their analysis could indirectly provide information about their environment, particularly if collected from sentinel species (Harley *et al.,* 2016). For instance, impaired immune activity of harbor seals has been related to high levels of organic pollutants (De Swart *et al.,* 1996).

Lymph node immune cell subsets have been described for animal models (Mason *et al.,* 1981) and humans (Poppema *et al.,* 1981, Fehniger *et al.,* 2003) and include T lymphocytes and NK cells. Interestingly, whereas experiments with T cells have been carried out quite frequently, the activity of NK cells isolated from lymph nodes has not been as widely studied, especially for marine mammals (Ross *et al.,* 1996). NK cells are relevant to marine mammal studies, as they are involved with early oncovigilance and viral surveillance (Beyer and Schultze, 2006; Ruffell *et al.,* 2010). The establishment of an *in vitro* NK cell assay could help to understand their role in the development and epidemiology of specific diseases of concern for wild marine mammals, such as California sea lion urogenital carcinoma (see Gulland *et al.,* 1996, Browning *et al.,* 2015), morbillivirus outbreaks (Duignan *et al.,* 2014, Venn-Watson *et al.,* 2015, Ludes Wehrmeister *et*

al., 2016) and other viral diseases (Fereidouni *et al.,* 2016), which could be prevalent in the marine environment (Culley *et al.,* 2003).

In this chapter, I examined whether lymph node mononuclear cells (LNMC) isolated from California sea lions display NK cell activity against tumor cells. This information was essential to be able to conduct assays where cytotoxicity and proliferation would be examined under exposure to contaminants (see Chapter 5).

4.2 Methods

4.2.1 Sample collection and cell harvesting

To conduct preliminary trials, mesenteric lymph nodes were harvested from two fresh CSL carcasses that died or were euthanized due to poor clinical prognosis while in rehabilitation at The Marine Mammal Center following stranding along the Central California coast. Samples of lymph node were collected aseptically, placed in complete Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) and shipped on an icepack to the laboratory (University of Connecticut, US) to be processed within 24 hours of collection. Sampling was conducted under MMPA permit 18786 issued to The Marine Mammal Center (TMMC, Sausalito, CA, USA).

Lymph node samples were stored in complete Dulbecco's Modified Eagle's Medium (see below) and placed on cold packs for shipping. Upon arrival, the lymph nodes were sliced into several sections of ≤ 1 cm^3 and cells were extracted by repeated flushes with Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Grand Island, NY) using a sterile 10 ml syringe and

23G needle. Erythrocytes were lysed using NH_4Cl (Levin *et al.,* 2007) and mononuclear cells were isolated using Ficoll-Paque 1.077 (GE Healthcare Life Sciences, Pittsburgh, PA) at 990 g for 40 minutes prior to re-suspending in complete Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 1 mM sodium pyruvate, 100 µM non-essential amino acids, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM l-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) (all from Gibco, Grand Island, NY) and 10% fetal calf serum (HyClone, GE Healthcare Life Sciences, Pittsburgh, PA). Cells were cryopreserved at 107 cells/mL in fetal calf serum with 10% dimethyl sulfoxide (DMSO, Sigma, St Louis, MO), progressively cooled overnight to -80˚C in a CoolCell® cell freezing container (BioCision, San Rafael, CA), and transferred to a liquid nitrogen storage container, where they remained for at least one month prior to the assays being performed.

4.2.2 Measurement of NK cell-like activity

Cells were quickly thawed in a 37ºC bath and immediately added to 50 mL tubes containing warm complete DMEM. Tubes were centrifuged and washed with DMEM twice, and viability was assessed with Trypan blue and light microscopy. Viability was calculated as [# live cells / (# dead cells + # live cells)] x 100, and was greater than 80% for both sea lions.

I used K-562 (CCL-243™, ATCC, Manassas, VA), a human myelogenous leukemia cell line and YAC-1 (TIB-160™, ATCC, Manassas, VA), a murine lymphoma cell line, as targets for LNMC cytotoxicity. Both target cell lines are recognized as generally sensitive to NK cytotoxic activity (Kiessling *et al.,* 1975), and have been used to assess cytotoxicity in cetaceans and

pinnipeds (de Guise *et al.,* 1997; Levin *et al.,* 2005; Ross *et al.,* 1996, Gebhard *et al.,* 2015) and other wildlife species (Rousselet *et al.,* 2013, Deforges *et al.,* 2018). Target cells were cultured in complete DMEM, supplemented with (all from Gibco, Grand Island, NY) 1 mM sodium pyruvate, 100 µM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, along with 10% FCS), at 37°C with 5% CO2 as per ATCC's recommendation.

NK cell-like activity was measured using the mortality of target cells at different LNMC to target cell ratios using flow cytometry as previously described (Deforges *et al.,* 2018). Briefly, 1 mL of target cells (K-562 or YAC-1) were incubated with 10 µL of 3 mM 3,3'-dioctadecyloxabocyanine perchlorate (DiO, Molecular Probes, Grand Island, NY) dissolved in DMSO. Cells were incubated for 20 minutes at 37˚C and 5% CO_2, washed with DMEM and adjusted to 10^5 cells/mL. The CSL LNMC used as effector cells were adjusted to 10^6 cells/mL, and 1, 0.5, 0.250, 0.125 and 0 mL of this preparation were pipetted into different 5 ml round bottom tubes. The viability of LNMC was above 80% for these samples. The volume of the tubes was completed with DMEM to reach 1 mL and 100 µL of K-562 or YAC-1 cells were added to achieve effector:target (E:T) ratios of 100:1, 50:1, 25:1, 12.5:1. A tube that contained target cells alone was used as a control to assess spontaneous mortality. The E:T mixtures were centrifuged for 30 seconds at 220 g and then incubated for 150 minutes at 37˚C and 5% CO_2. All tests were performed in duplicate.

Following centrifugation at 220 g for 10 minutes at 4˚C, the supernatant was discarded and the cells were re-suspended in 200 µL of phosphate buffer saline solution and placed on wet ice before immediate analysis. A solution of 50 µg/mL of propidium iodide (PI, Molecular Probes, Eugene, OR) was added to each tube immediately prior to acquisition to evaluate mortality of the

target cells using two-color (DiO *vs.* PI) flow cytometry. The fluorescence of at least 1000 target cells was read using a FACScan flow cytometer (Becton Dickinson, Rutherford, NJ) and the automated CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA). Effector cells were identified by their relative size (forward-scattered light, FSC) and their complexity (side-scattered light, SSC) and distinguished from DiO-labeled target cells, which show higher fluorescence at 530 nm (FL-1). Dead or dying cells incorporate PI due to membrane instability, and they showed higher fluorescence at 630 nm (FL-3). I selected this method as it has been validated to measure innate cytotoxicity in some cetacean and pinniped species (de Guise *et al*., 1997; Levin *et al*., 2005; Ross *et al*., 1996). The percent target cell mortality was calculated as: [# dead target cells / (# dead target cells + # live target cells)] x 100. The percent of spontaneous target cell mortality was subtracted from the percent target cell mortality to calculate specific target cell mortality.

4.2.3 Statistical analyses

For the proliferation assay I used the Stimulation Index to compare proliferation using repeated-measure one-way analysis of variance (RM ANOVA) with a *post-hoc* Dunnett's test if p-values

were below 0.05. For data that did not pass normality and homocedasticity tests, the analysis was done using RM ANOVA on Ranks (Gholib *et al*., 2017). All analyses were run on SigmaStat 3.5.

For the innate cytotoxicity assay I used RM ANOVA with Dunnett's *Post hoc* test. For normality, I used Shapiro-Wilk test and equality of variance was assessed by a Brown-Forsythe test (Brown and Forsythe, 1974).

4.3. Results

There was a consistent pattern of decreased specific mortality with decreasing E:T ratios with both YAC-1 and K-562 cell lines, suggesting that both cell lines were vulnerable once spontaneous lysis was subtracted (Fig. 4.1). YAC-1 cells were approximately three times more sensitive to CSL LNMC cytotoxic activity than K-562 cells (μ=10.12%, SD=4.72 and μ=3.92%, SD=0.26 for 100-1 effector:target ratio, respectively; μ=10.47%, SD=4.38 and μ=2.99, SD=0.03 for 50-1; μ=7.8,4 SD=4.87 and μ=3.42, SD=0.13 for 25-1; μ=8.23% SD=4.04 and μ=1.39 SD=0.32 for 12-1). In all cases, spontaneous lysis remained under 4%.

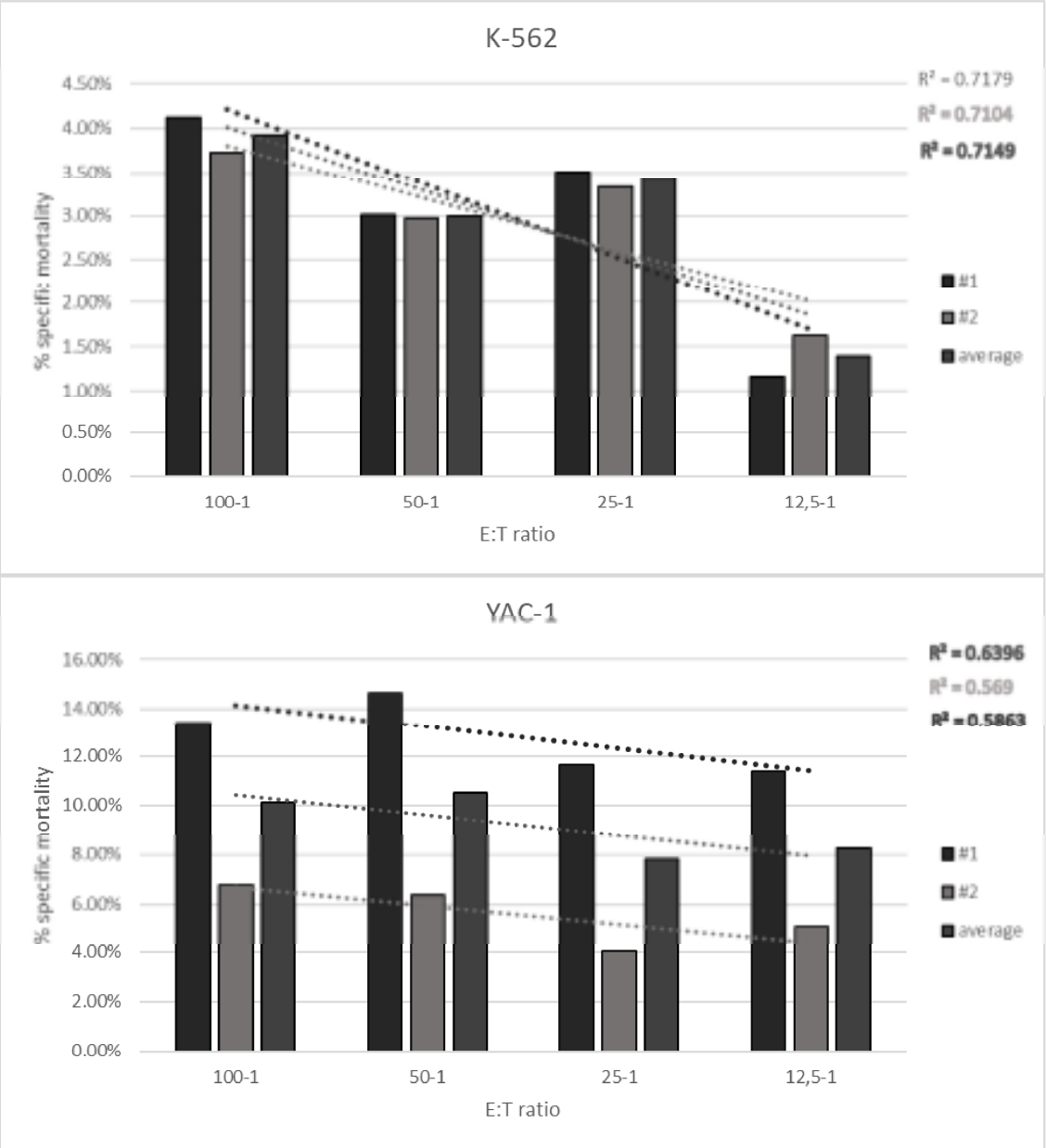

Figure 4.1 *In vitro* mortality of K-562 and YAC-1 after exposure to CSL LNMC. The x-axis shows the effector:target (ET) ratios used (100-1; 50-1, 25-1; 12.5-1). The black bars show % of specific mortality for LNMC from individual 1 (CSL 12454) and the light grey bars show % of specific mortality for LNMC from individual 2 (CSL 12560). The dark grey bar show the average for both individuals. In all cases, spontaneous lysis was subtracted. Bars indicate ± S.D.

4.4 Discussion

The fact that CSL LNMC showed higher sensitivity to YAC-1 than to K-562 concurs with previous observations in phocids (Ross et al., 1996; M. Levin, personal communication), and suggests that YAC-1 are appropriate target cells to use in future experiments with pinniped LNMC while K-562 cells would be more suitable for assays with cetacean LNMC (de Guise *et al.,* 1997). It also suggests that, as has been shown in some studies (Callery *et al.,* 1980), even if decreased by extended periods of cryopreservation, LNMCs retain cytotoxic activity.

To the best of my knowledge, this is the first time that LNMC harvested from non-captive otariid pinnipeds have been used for cytotoxicity assays. In particular, this is the first to provide evidence that CSL LNMC exhibit *in vitro* innate cytotoxicity even after being cryopreserved for more than a month. Such *in vitro* models can provide essential information about cell physiology and innate cytotoxicity in non-model animals (Demas *et al.,* 2011). Furthermore, the fact that LNMC can be harvested in large quantities from fresh carcasses would allow multiple cytotoxicity experiments to use LNMC from one animal in order to eliminate inter-individual variability (see Mata *et al.,* 2014). However, any extrapolation of our findings to other marine mammal species should be taken with caution. Although it would be expected to observe cytotoxicity of LNMC across mammals, in some instances activity is minimal or undetectable, as has been observed in studies that used congenitally athymic nude *rnu*/+ rats (de Jong *et al.,* 1980). Interestingly, NK cells harvested from human lymph nodes usually lack perforin (Vivier *et al.,* 2008), so they do not display innate cytotoxicity (Strbo *et al.,* 2003). Based on these observations, it is possible that CSL LNMC synthesize perforin. Whether this is a common finding across marine mammals remains to

be seen, and future studies should check cells for cytotoxic activity before using this assay in other species

It is not the first time that cryopreserved cells have been used for cytotoxicity experiments. For example, cytotoxicity of cryopreserved cells collected from wild animals have been assayed with Cr51, but these assays relied on PBMC isolated from blood samples and used within 24 hours from collection (see de Guise *et al*., 1997). This is the first time, to the best of my knowledge, that LNMC have been extracted and used for cytotoxicity assays. Also, while innate cytotoxicity has been shown for NK cells harvested from murine and human lymph nodes, this is the first attempt to confirm such cytotoxicity in cells extracted from a lymph node of a pinniped and, particularly, from an otariid. In mice and human lymph nodes, NK cells usually lack perforin (Vivier *et al*., 2008), so they are not supposed to exert innate cytotoxicity (Strbo et al., 2003). Although it can be expected to find cytotoxicity in lymph nodes across mammals, in some cases, such as rnu/+ mice, activity is minimal or undetectable (de Jong *et al*., 1980). Thus, it would be necessary to check for cytotoxic activity in any models before asserting it.

In conclusion, I have shown that cryopreserved cells extracted from lymph nodes collected from CSL fresh corpses display innate cytotoxicity. Furthermore, I have established a model for future toxicology assays in animals from which collection of blood samples from live animals can be complicated, and have shown that cells remain viable for at least one month after collection. This work constitutes a new approach for immunotoxicology assays of wild animals.

5. PROLIFERATION AND CYTOTOXICITY OF CSL LNMC UNDER ENVIRONMENTALLY-RELEVANT ORGANOCHLORINE CONCENTRATIONS

Note: The research presented in this chapter led to the publication of a peer-reviewed manuscript published by Environmental research (accepted on August, X, 2018) (Peñín-Fernández, I., Levin, M., Acevedo-Whitehouse K., Jasperse, L., Gebhard, E., Gulland, F.M.D., De Guise, S. (in press). Effects of polychlorinated biphenyls (PCB) on California sea lion (Zalophus californianus) lymphocyte functions upon in vitro exposure. The paper is included in Annex I.

5.1 Introduction

The increase in the number of unusual mortality events and strandings, as well as a marked rise in cases of cancer (Browning *et al*., 2015) have highlighted the importance of understanding the role of persistent organic pollutants (POPs), such as organochlorines, on marine mammal health (Brown *et al*., 2018) This is because contaminants are involved in immunomodulation (Ross *et al*., 1996; de Swart *et al*., 1996), and can directly cause some pathologies (Desforges *et al*., 2016). Furthermore, POPs have been associated with the development of tumors in Beluga whales, *Delphinapterus leucas* (de Guise *et al*., 1995). Among this kind of pollutants, chlorinated organic pollutants, such as PCB congeners (polychlorinated biphenyls) and DDT metabolites (dichloro diphenyl trichloroethane) have been reported to induce, in a concentration dependent manner, pathological states in animals (Ross *et al*., 1996).

Organochlorines have been detected in the tissues and blood of various marine mammal species (Luckas *et al*., 1990; Lahvis *et al*., 1995; Krahn *et al*., 1997; Kajiwara *et al*., 2001; Desforges *et al*., 2016). Among the adverse effects that these congeners and their metabolites cause to marine mammals, are decreases in serum levels of vitamin A, diminution of reproductive success, and reduced production of thyroid hormone in harbor seals, *Phoca vitulina* (Ross *et al*.,

1996); induction of premature birth in CSL (Delong *et al*., 1973), and immunomodulation in various species (Mori *et al*., 2006b, 2008b). Carnivorous marine mammals are top predators, and, thus, are particularly vulnerable to POP-driven effects, as they are exposed to high concentrations of hidrophobic POPs due to bioaccumulation along the food chain (Ross, 2002). Also, as top predators, these animals are in the same trophic web position as humans, even feeding in the same fisheries (Ross, 2002) which makes them particularly useful sentinels to detect potential harm to human population despite their obvious physiological differences (see Mori *et al*., 2006).

Recent studies conducted in humans have lead the IARC to determine the role of organochlorines as probable or possible carcinogens (Baan *et al*., 2009) and even, dioxin-like polychlorinated biphenyl compounds (PCBs) have been classified as carcinogenic to humans based on laboratory models and on results obtained by epidemiological research (Lauby-Secretan *et al*., 2013). The role of PCBs in carcinogenicity is thought to be related to the Aryl Hydrocarbon Receptor (AhR), a ligand-activated transcription factor involved in the regulation of biological responses to planar aromatic hydrocarbons, but there is evidence that this is not the only mechanism of action (Lauby-Secretan *et al*., 2013).

Although the use of PCBs has been banned in USA by the Environmental Protection Agency since the 1970s, and in most countries since the 1980s (Lauby-Secretan *et al*., 2013), and DDT is barely used except in the developing world (Kabasenche and Skinner, 2014) mainly for vector control (Raghavendra *et al*., 2011), marine mammal populations from Californian waters, particularly CSLs, still have a high concentration of these pollutants in comparison with populations from other regions (Ylitalo *et al*., 2005; Del Toro *et al*., 2006; Niño-Torres *et al*., 2009). These pollutants magnify through the trophic web, and accumulate in the blubber of marine mammals (Tanabe, 2002). The impact of PCBs and other organochlorines on the CSL immune

system has been proposed based on correlational data, but a mechanism has not been revealed yet (Ylitalo *et al*., 2005). Given that the immune system is one of the most important physiological effectors that determines the fate of an individual's interaction with its environment, its characterization could provide useful information on the impact of the effects that organochlorines can have on animal health.

The assessment of lymphocyte proliferation is a tool traditionally used to measure the status of the immune system and has been used in different marine mammal species prior to this study (Lahvis *et al*., 1995; de Guise *et al*., 1998; Levin *et al*., 2005; Mori *et al*., 2006). Prior to those studies, a tool named TEQ (Toxic equivalent) was developed using mice as a model to predict the effects of organochlorines. TEQ was shown to be incapable of predicting organochlorine effects in some marine mammals as the predicted additive effects work for mice but not for other species (Mori et al. 2006). These effects are caused by dioxin-like PCB congeners (co-planar PCBs), but non-coplanar PCBs have been shown to affect proliferation *in vitro* in some marine mammals (Levin *et al*., 2005; Mori *et al*., 2006). Individual PCBs and mixtures of these compounds harm T- and B-cell proliferation and immunoglobulin production in the bottlenose dolphin, *Tursiops truncatus* (Lahvis *et al*., 1995; Mori *et al*., 2008). In pinnipeds, T- and B-cell proliferation was correlated to levels of PCB congeners in the harbour seal (Levin *et al*., 2005).

To date, the effects of PCB on NK cytotoxicity are less clear, as experiments carried out in harbor seals have shown contradictory results, plausibly due to different experimental designs. Seals fed on a diet laced with high levels of pollutants showed a significant decrease in cytotoxicity (de Swart *et al*., 1994; Ross *et al*., 1996). In contrast, *in vitro* analyses of mononuclear blood cells (PBMC) proliferation, showed no differences in grey and harbour seals when their blood cells were exposed to different concentrations of a commercial PCB mixture (Hammond *et al*., 2005).

Despite the differences found for NK cytotoxicity, *in vitro* studies found that after short periods of time (24 hours of exposure), other innate immune functions, such as respiratory burst and phagocytosis, were affected by the pollutants (Hammond *et al*., 2005). Moreover, otariid and phocid lymphocyte populations have been shown to behave in different ways when exposed to similar concentrations of pollutants (Mori *et al*., 2006, 2008).

The CSL has a large population and a wide distribution, ranging from the Pacific coast of Canada and along the Pacific USA coastline down to Mexico, where the species is relatively abundant. The species has been well studied at different levels, from behavioral (e.g. Feldkamp *et al*., 1989), genetic (e.g. Maldonado *et al*., 1995; Acevedo-Whitehouse *et al*., 2003a; Bowen *et al*., 2004; Schramm et al. 2009; Browning et al. 2015) demographic (e.g. Aurioles et al. 1983; Le Boeuf 1983; Szteren *et al*., 2006), epidemiological (e.g. Greig *et al*., 2005; Goldstein *et al*., 2008) or toxicological approaches (Lefebvre *et al*., 1999; Stapleton *et al*., 2006). Having such a wealth of information for a free-living marine mammal is uncommon; however, studies on the interaction between PCB and DDT and its immune response have yet to be conducted. This omission is particularly interesting as the CSL is affected by urogenital carcinoma (Browning *et al*., 2015), one of the rare cases of metastatic cancer in wildlife, and a correlative link to high PCB levels in the blubber of dead stranded CSL afflicted with urogenital carcinoma has been reported (Ylitalo *et al*., 2005).

As occurs for most types of cancer, urogenital carcinoma appears to have a multifactorial origin, including, in addition to organochlorines, a potentially-oncogenic gammaherpesvirus (King et al. 2002), genital bacteria (Johnson *et al*., 2006), high levels of inbreeding (Acevedo-Whitehouse *et al*., 2003a), and genetic variants (Bowen et al. 2005; Browning and Acevedo-Whitehouse *et al*., 2014). The role of organochlorines warrants further exploration, as there have

been no reported cases of urogenital carcinoma in CSL from the Gulf of California, despite evidence of c cellular transformation of the genital tract (Barragán-Vargas *et al*., 2016). In this area of their distribution, organochlorine levels are between two and three orders of magnitude lower (Del Toro *et al*., 2006), so it is tempting to speculate that, indeed, organochlorines influence carcinogenesis or metastasis.

As the level of PCBs in free-ranging CSLs is below that reported to be directly carcinogenic in murine models (Silberhorn *et al*., 1990; Ylitalo *et al*., 2005; Del Toro *et al*., 2006; Blasius and Goodmanlowe, 2008), it is possible that the observed association with CSL urogenital carcinoma (Ylitalo *et al*., 2005) could arise via modulation of the immune response. Similar concentrations (5 ppm to 15 ppm of PCBs) drive immunomodulative responses in lymphoproliferation in various marine mammals, human and mice (Mori *et al*., 2006).

In this chapter, I investigated the effect of PCB congener exposure on NK cell and T cell activity using *in vitro* assays on cells harvested from CSL lymph nodes. To test whether environmentally relevant concentrations of PCBs immunomodulate the response of CSL NK cells and T cells *in vitro*, three approaches were used. First, LNMC were examined to see whether they displayed NK cell-like activity against tumor cells. Secondly, NK cell-like activity was examined under different concentrations of individual congeners, as well as a PCB congener mixture. Finally, lymphoproliferation assays were carried out at the same PCB concentrations used for the NK cell-like activity assays.

5.2 Methods

I designed two experiments to compare lymphocyte proliferation and innate cytotoxicity under different concentrations of individual PCB congeners, as well as a mixture of PCBs according with their common values in free-ranging California sea lions (Del Toro *et al*., 2006). For lymphocyte proliferation, I also performed an assay with different DDE concentrations.

5.2.1 Sample collection

Mesenteric lymph nodes were harvested from 19 fresh CSL carcasses and sent to the laboratory at the University of Connecticut to be processed within 24 hours of collection. Sampling was conducted under MMPA permit 18786 issued to The Marine Mammal Center (TMMC, Sausalito, CA, USA). All samples were collected from animals undergoing rehabilitation at TMMC. After collection, lymph nodes were shipped to the University of Connecticut in sterile cryovials containing fresh complete Dulbecco's modified Eagle medium (DMEM). Complete medium was prepared with DMEM supplemented with 1mM sodium pyruvate, 100 uM nonessential aminoacids, 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin, Fungizone, and 10% Fetal Calf Serum (FCS). Once the lymph nodes arrived at the laboratory, they were prepared as indicated in Chapter 4.

Owing to contamination of six samples, LNMC from 13 CSL were extracted and cryopreserved. To determine the number of samples needed, I assumed two points of difference of means, according with preliminary results and 2 points of standard deviation. With a beta error of

0.2 and an alpha error of 0.05, the number of animals needed to reach the desired statistical power was 7.85 animals. Thus, the sample size for each treatment was eight, unless a statistical power of 0.80 was achieved using fewer animals.

5.2.2 Measurement of NK cell-like activity under exposure to organochlorine pollutants

Although *in vitro* assays have been described to characterize NK cell activity, given the description of NK cell-like cytotoxicity by gamma-delta T cells (Lilienfeld-Toal *et al.,* 2006) and other innate lymphoid cell subsets (Tian *et al.,* 2016), and the use of mixed and uncharacterized cell types from sampled CSL lymph nodes, in this Chapter I refer to the description of NK cell-like cytotoxicity. The viability of the LNMC was above 80% for all samples used.

As PCB congeners are commonly found as mixtures in the environment (Robertson and Hansen, 2015), I tested individual congeners that have previously been detected in blubber of wild CSL (dioxin-like congeners 105, 118 and 169, and non dioxin-like congeners 138, 153 and 180) (Ylitalo *et al.,* 2005; Del Toro *et al.,* 2006), as well as a mixture of congeners, that was prepared using the relative proportions of the selected pollutants reported for wild CSL (Del Toro *et al.,* 2006). For each PCB congener, three different biologically relevant concentrations (5 ppm, 10 ppm, 15 ppm in 0.4% DMSO) were tested based on values reported in CSL blubber (Ylitalo et al., 2005; Del Toro et al., 2006), in addition to an unexposed control of 0.4% DMSO alone.

A 100 µL volume of thawed CSL LNMC (2×10^5) was pipetted into 5 ml round bottom tubes (see Chapter 4 for procedural details on cell handling). Individual PCBs or the mixture, as well as complete DMEM, was added to reach a final volume of 200 µL. The tubes were incubated for 3 h

at 37˚C with 5% of CO2. DiO-labeled YAC-1 cells were added at a final E:T ratio of 50:1, after having determined this to be the ideal E:T (see Chapter 4). Cells were incubated for 150 min, centrifuged, and resuspended in fresh DMEM. Target cell fluorescence was acquired on a FACScan flow cytometer as described earlier. All assays were conducted in duplicate.

5.2.3 Lymphocyte proliferation assay

Concanavalin A (ConA)-induced lymphocyte proliferation was carried out similar to assays previously performed in lymphocytes harvested from pinnipeds and other marine mammals (Mori *et al.,* 2006). For each treatment, cryopreserved LNMCs were thawed, washed twice in complete DMEM and adjusted to 2 x 10^6. Cells were plated in triplicate in 96 flat bottom wells plates (Falcon, Becton Dickinson, Franklin Lakes, NJ). Two concentrations of ConA, an optimal concentration at 1 µg/mL and a suboptimal concentration at 0.1 µg/mL, were used. The suboptimal mitogen concentrations were selected because they have been shown to be more sensitive at detecting immunotoxicity (Mori *et al.,* 2006).

Cells were exposed to the same three concentrations of individual PCB congeners and PCB mixture as described for the NK cell-like activity assays. Cells were incubated at 37˚C with 5% of CO_2 for 48 hours before adding BrdU, a thymidine analogue, in order to assess proliferation. Cells were incubated for an additional 18 hours and BrdU was detected with a monoclonal antibody and a colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU [colorimetric], Roche Diagnostics, Alameda, CA), as per the manufacturer's instructions. Plates were read with an ELISA plate reader (Multiskan EX v.1.0) at 690 nm with a reference wavelength of 450 nm. The

triplicate measurements of the optical densities were averaged and the Stimulation Index was calculated by dividing each result by that of the unstimulated control sample (see Neckers and Cossman, 1984; Bartholomew *et al*., 2002). All assays were conducted in triplicate.

In order to assess the effect of DDT on proliferation, first I had to establish that methanol did not impact lymphocyte proliferation at 5%., as DDT is eluted in methanol. This was indeed the case; when testing for cytotoxicity (83% of proliferation against control, p=0.037), and, thus, I added methanol to the 0 ppm in order to control potential effects. Preliminary DDE results showed no differences when n=3 (RM ANOVA; $F_{3, 4.690}$; p=0.051;) for suboptimal concentration and for optimal when n=4 (ANOVA on ranks; Chi-square= 5.615 with 3 degrees of freedom. P(est.)= 0.132 P(exact)= 0.141), and proliferation decreased when DDE concentration increased. As results of proliferation under DDE were unreliable due to the cytotoxicity induced by methanol, the data could not be considered biologically significant, and I discarded these experiments.

All data were analysed before statistical analyses to confirm they were responding properly to the mitogen properly. Some samples did not respond to mitogen at all (their stimulation index for suboptimal and optimal mitogen neared 1) and others responded excessively (stimulation index values above 8). I repeated those samples to have a representative population and to avoid adding noise to the experiment.

5.2.4 Statistical analysis

Shapiro-Wilk tests were used to determine deviations from normality, and equality of variance was assessed by a Brown-Forsythe test (Brown and Forsythe, 1974). Repeated-measure analysis

of variance (RM ANOVA) and post-hoc Dunnett's tests were used to determine if each PCB concentration was significantly different from the unexposed control. For results that deviated from assumptions of normality, data were normalized by a square root transformation. If, despite transformation, the data did not show evidence of normality and homoscedasticity, data were analyzed using RM ANOVA on Ranks (Gholib *et al.,* 2017). All analyses were run on SigmaStat 3.5 (Systat software, Inc.) and graphs were created using GraphPad Prism 7.04 for Windows (GraphPad Software, La Jolla California USA). For all tests, statistical significance was established at $p<0.05$.

5.3 Results

5.3.1 NK cell-like activity under exposure to PCBs

NK cell-like activity was not affected significantly by four of the six individual PCB congeners when compared to the control (RM ANOVA; PCB 105, $F_{3,8}=2.160$, p= 0.123, Fig. 5.1a; PCB 118, $F_{3,8}=1.618$, p= 0.215, Fig. 5.1b; PCB 153, $F_{3,8}=2.722$, p= 0.070, Fig. 5.1d, and PCB180, $F_{3,8}=0.808$, p= 0.504; Fig5.1f). PCB 169 decreased NK cell-like activity by 5.2% at 15 ppm (RM ANOVA; $F_{3,8}=3.327$, p=0.039, Fig. 5.1e). Also, there was a non-significant trend suggestive of a concentration-dependent increase in NK cell-like activity for PCB 138 (RM ANOVA; $F_{3,8}=2.929$, p= 0.054; Fig. 5.1c). The experiment that used the PCB mixture showed decreased NK cell-like activity of lymphocytes at 15 ppm when compared with 0 ppm (RM ANOVA on Ranks; $Chi^2=$ 8.250, df=3, p =0.041; Fig. 5.1g).

5.3.2 Innate cytotoxicity assay

For optimal mitogen concentrations, non dioxin-like congeners 153 (at 5 and 10 ppm) and 180 (at 5 and 10 ppm) and dioxin-like congener 105 (at 5 ppm) showed increased lymphocyte proliferation, and non dioxin-like PCB 138 showed a decrease at the highest (15 ppm) concentration (RM ANOVA; PCB 105, $F_{3,5}$ =5.869, p=0.007; PCB 153, $F_{3,5}$=22.516, p<0.001; PCB 180, $F_{3,4}$=6.853, p=0.011; PCB 138, $F_{3,8}$=5.224, p=0.007; Fig. 5.2a). Although PCB 118 behaved similar to the other congeners analyzed, and showed significant differences among concentrations (RM ANOVA; PCB 118 $F_{3,8}$=3.332, p=0.039; Fig. 5.2b), the *post hoc* Dunnett's test showed no significant difference from unexposed control. PCB 169 did not induce significant changes in lymphocyte proliferation (RM ANOVA; $F_{3,8}$=2.247, p=0.113; Fig. 5.2e). Proliferation of lymphocytes exposed to the PCB congener mix at optimal mitogen concentration varied significantly among concentrations (RM ANOVA on RANKS; Chi^2= 12.3, df=3, p=0.006; Fig. 5.2g), although the *post hoc* test showed no significant difference from unexposed control.

Suboptimal mitogen concentration (Fig. 5.3), which according to Mori et al., (2006) was considered to be more sensitive to demonstrate immunomodulatory effects in marine mammals, showed similar results to those observed for optimal mitogen concentration. The exception was congener 105, which show a non-significant trend of concentration-dependence (ANOVA on ranks; Chi^2= 6.45, df= 3, p = 0.092; Fig. 5.3a). PCB 118 and PCB 169, the other two dioxin-like congeners, showed no difference at suboptimal mitogen concentration (PCB 118, ANOVA on ranks; Chi^2= 2.316, df= 3, p = 0.509; PCB169, RM ANOVA, $F_{3,7}$=1.170, p=0.339). Non dioxin-

like congeners showed results that were consistent with those found for the optimal mitogen stimulation (RM ANOVA; PCB 138, $F_{3,8}$ =2.719, p= 0.070; PCB 153, $F_{3,5}$ =22.516, p<0.001; PCB 180, $F_{3,4}$ =6.853, p=0.011). Although PCB 138 did not vary significantly, the observed pattern was similar to what was found for optimal mitogen concentration; PCB 153 induced differences for both 5 and 10 ppm; and 180 was different at 5 ppm, but not at 10 ppm). Proliferation of lymphocytes exposed to the PCB congener mix at suboptimal mitogen concentration did not show any difference between treatments (RM ANOVA; $F_{3,7}$= 1.237, p=0.321; Fig. 5.3g).

5.4 Discussion

The results presented in this chapter were based on experimental *in vitro* assays, and as such, does not necessarily imply that an organism would respond similarly under natural exposure to contaminants. However, the environmentally-relevant concentrations used could be considered a good model to understand the effects exerted by organochlorines on CSL NK and T-cell activity, and especially, on oncosurveillance (Canning *et al*., 2006; Carayannopoulos and Yokoyama, 2004). If the activity of these immune cells is modulated by organochlorines, it is plausible to consider that infection by oncoviruses, such as OtHV-1 (Lipscomb *et al*., 2000), and tumorigenesis are facilitated in exposed CSL.

NK cell-like activity was not affected significantly by four of the six individual PCB congeners when compared to the control. However, PCB 169 decreased NK cell-like activity by slightly more than 5.2% at 15 ppm. This reduction in NK cell-like activity is similar to the previous findings in phocids fed with organochlorine polluted herring (Ross *et al*., 1996b).

The biological significance of this effect is unknown, as dioxin-like PCB congeners are underrepresented in free-ranging CSL tissues (Del Toro *et al*., 2006), however, as stated earlier, these can accumulate quickly in nursing pups (Sørmo *et al*., 2003). Unfortunately, the NK cell-like cytotoxicity assays were mostly inconclusive due to the low number of samples available for analysis. However, despite this limitation, the assays showed very interesting trends that warrant being repeated with more samples in the future. The dioxin-like PCB congeners at low concentrations did not affect cytotoxicty. However, PCB 138, a non-dioxin-like congener, appeared to increase the cytotoxicity in a concentration dependent manner, reaching the highest values of cytotoxicity at 15 ppm concentration. This is consistent with cell assays conducted in invertebrate animal models (Belmeskine *et al*., 2012), and could imply that the non-dioxin-like congeners exert their action via different (as yet unknown) mechanisms than dioxin-like congeners. In other studies, when animals were fed a diet of herrings from polluted zones, NK activity decreased, but the molecular mechanism responsible for the effects was not identified (Ross *et al*., 1996). Regretfully, the result was not statistically significant as there was limited power to detect differences (the statistical power remained below 0.4 for all congeners). If the pattern were confirmed using more samples, this intense response of the innate cytotoxicity at this pollutant concentrations can drive to anergy status or, if continued in time, to autoimmune diseases.

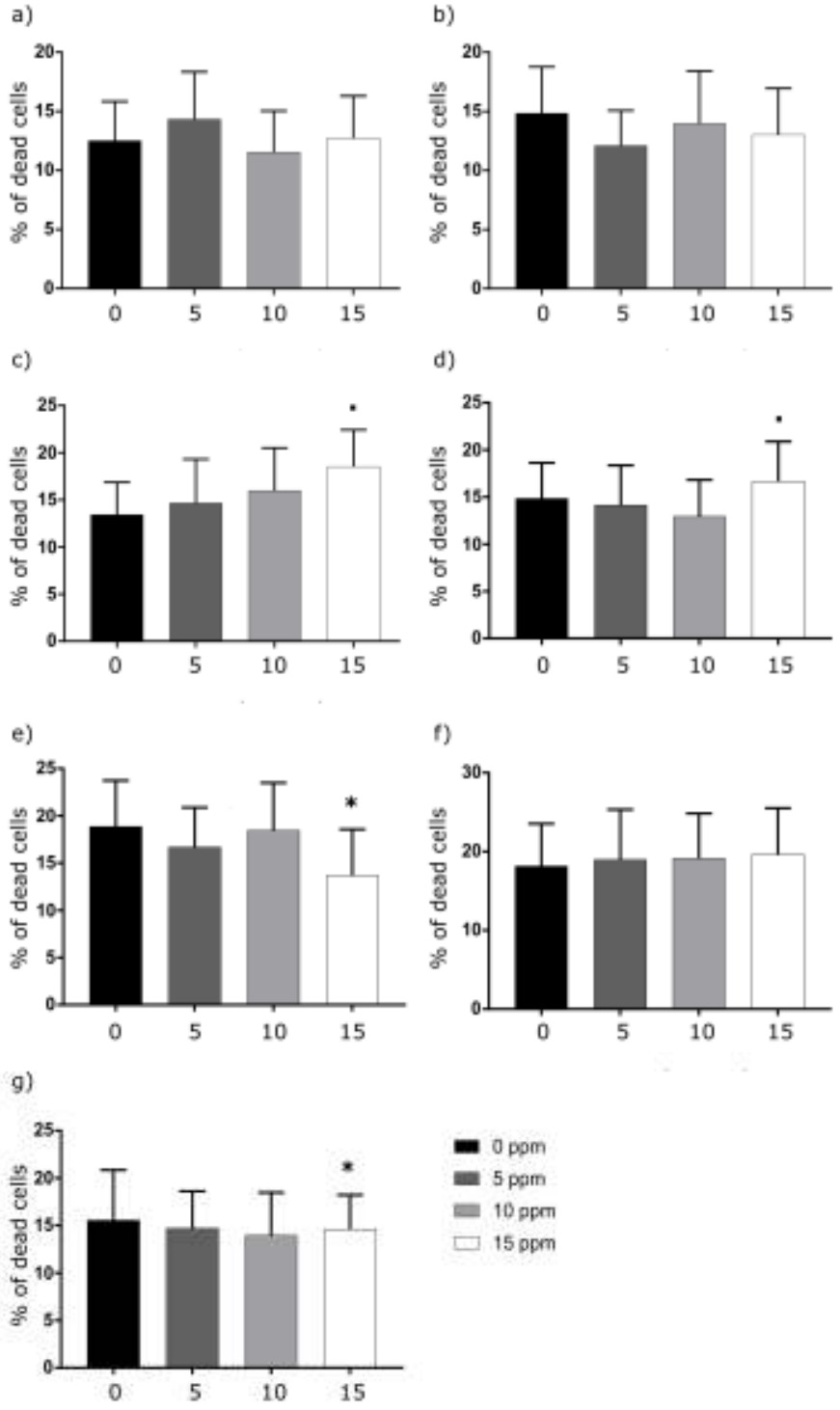

Figure 5.2 *In vitro* mortality of YAC-1 cells by CSL LNMC exposed to different concentrations of PCB congeners.
The effector:target ratio was 50:1). a) PCB 105, b) PCB 118, c) PCB 138, d) PCB 153, e) PCB 169, f) PCB 180, g) PCB mix. Bars indicate ± S.E. * indicates $p<0.05$, □▪ indicates $p<0.1$.

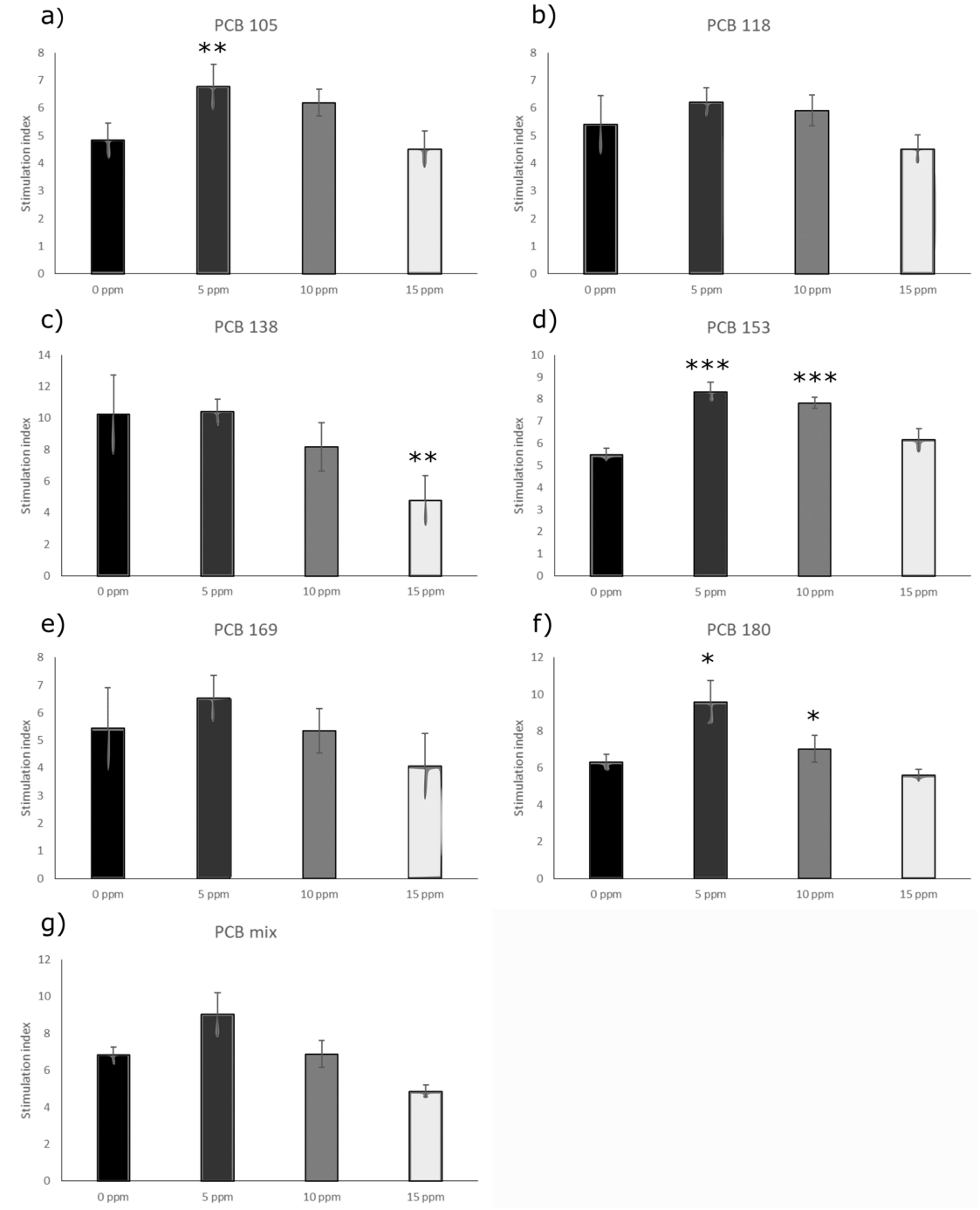

Figure 5.3 Proliferation of LNMC exposed to PCB congeners under optimal stimulation (1 µg/mL) of ConA.

a) PCB 105, b) PCB 118, c) PCB 138, d) PCB 153, e) PCB 169, f) PCB 180, g) PCB mix. Proliferation was measured as the Stimulation Index. Bars indicate ± S.E. *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$.

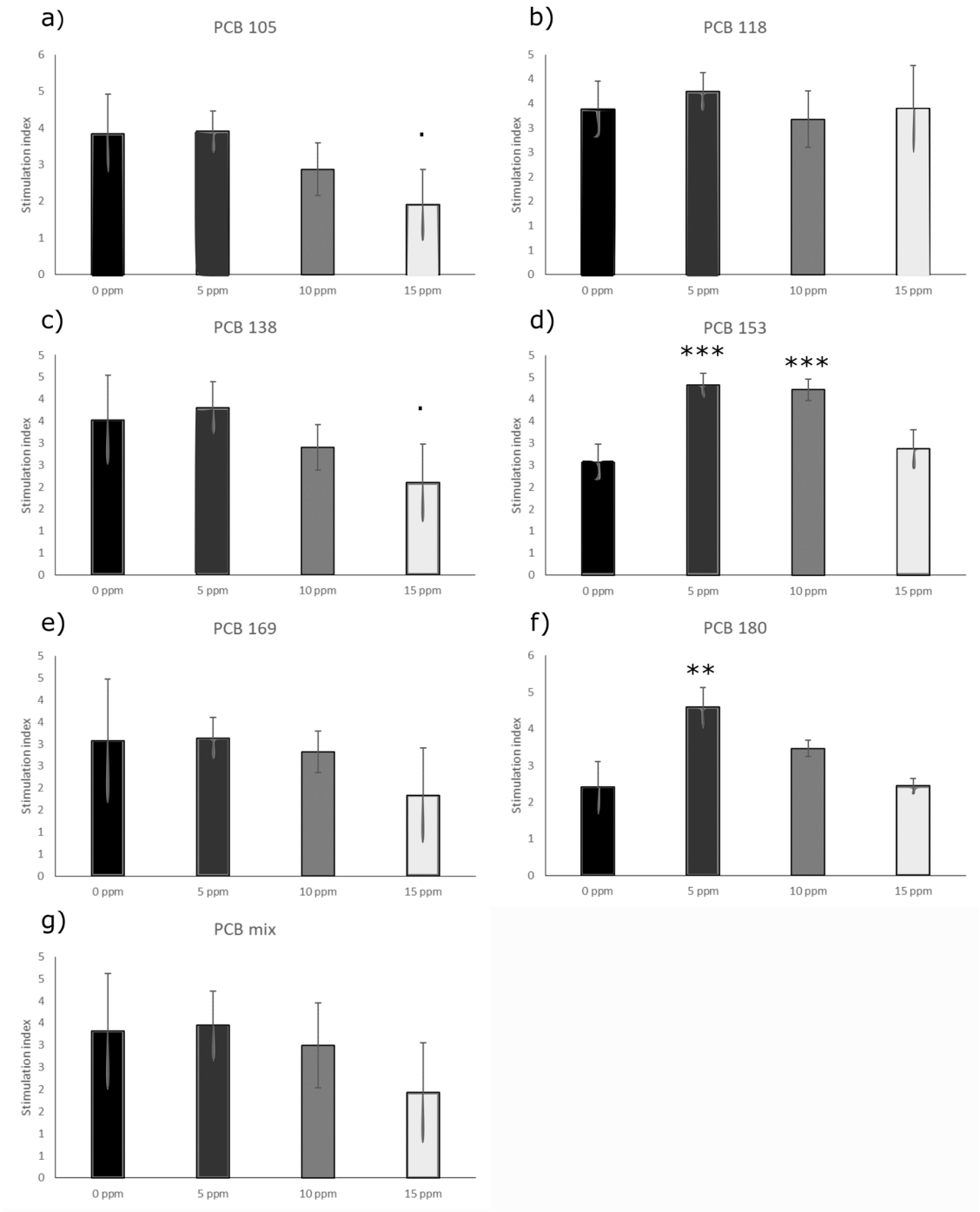

Figure 5.4 Proliferation of LNMC exposed to PCB congenersunder suboptimal stimulation (0.1 µg/mL) of ConA.
a) PCB 105, b) PCB 118, c) PCB 138, d) PCB 153, e) PCB 169, f) PCB 180, g) PCB mix. Proliferation was measured as the Stimulation Index. Bars indicate ± S.E. *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$.

In terms of proliferation, based on my results, it appears that the effects of both types of PCB congeners (dioxin-like *vs.* non-dioxin-like) on CSL lymphocytes differs. While, except for PCB138, non-dioxin-like congeners seemed to induce proliferation of immune cells when at low concentrations, while a clear pattern of concentration-dependent suppression was observed for a dioxin-like congener (PCB 105) when using suboptimal mitogen concentration. It is interesting that a similar effect of PCB 138 was found for East Greenland ringed seal (*Pusa hispida*) at the same concentration as used here (Levin *et al.,* 2016). PCB 138 was previously found to be able to induce UGT1A6 transcription in rat hepatoma cells independently of the androstane receptor CAB, which is the mechanism by which non dioxin-like PCBs affect this gene transcript. In addition, UTGA1A6 is generally upregulated by the presence of dioxin-like PCBs via an AhR-dependent mechanism (Hamers *et al.,* 2011). Although it does not mean that PCB138 is a dioxin-like PCB instead of non dioxin-like, it may exert somehow an structural effect on AhR or maybe there are other unexplored cell routes to be described.

The potential relevance of these results warrants discussion. PCB 105 is typically low in free-ranging CSL blubber and serum (Del Toro *et al.,* 2006; Niño-Torres *et al.,* 2009; Ylitalo *et al.,* 2005). However, the observed effect should be considered carefully due to their mobilization in the organism. These types of low molecular weight, low chlorinated congeners are the first to be mobilized from the blubber to the milk and, thus, become more concentrated in neonatal pups, as has been seen in the grey seal, *Halichoerus grypus* (Sørmo *et al.,* 2003). If so, CSL pup lymphocytes could be restricted in their proliferative ability early in development. In addition, dioxin-like PCB congeners can activate the aryl hydrocarbon receptor (AhR) (Denison and Nagy, 2003; Mandal, 2005) and, if persistent, AhR activation could lead to chronic autoimmune

processes due to sustained Th17 responses, which are driven by Treg cells, and have been reported to occur in mice (Veldhoen *et al.,* 2008; Huo *et al.,* 2018). In addition, potential negative effects of the PCB congeners on lymphoid organs, such as the thymus or bone marrow, could be enough to drive the individual towards an anergic status (Couillard *et al.,* 2008; Repetto and Baliga, 1997), which could make CSL more susceptible to various pathologies, including urogenital cancer. However, before concluding anything from the assays, I must consider that some anti or proliferative may not be visible *in vivo*, as the non-dioxin-like congeners and other factors could hide this action.

Most of the PCB mixture was composed of non-dioxin-like congeners, as has been reported in the literature (Del Toro *et al*., 2006). It was interesting to notice that the data collected during the assays of CSL LNMC exposed to the PCB mix displayed a markedly non-normal behavior in terms of distribution and lack of homoscedasticity, both for innate NK cell-like activity and for proliferation at both mitogen concentrations. Plausibly, this behavior could reflect that when exposed to a mixture of dioxin-like and non dioxin-like congeners, CSL LNMC responses can be somewhat unpredictable (Levin *et al*., 2004; 2005a; 2005b), particularly because of complex additive, antagonistic and synergistic interactions that can occur between planar and non-planar PCBs (Mori *et al*., 2006). Alternatively, considering that the assays that used dioxin-like congeners 105 and 118 at suboptimal mitogen concentrations also displayed lack of normality and homocedasticity, these congeners could be driving the observed effects of the PCB mixture.

Based on the results of this study, it would appear that exposure to environmentally-relevant PCB concentrations either induces proliferation of CSL lymphocytes or does not cause any observable effect. Although these results defy the traditional view of organochlorines as immunosuppressive agents (Repetto and Baliga, 1997; Kerkvliet, 1995; Tryphonas *et al.,* 1991),

they concur with what has been reported for other pinnipeds (Mori *et al.,* 2006) that have shown that when at environmental concentrations, effects on immune cells tend to be proliferative. Organochlorines, particularly non dioxin-like congeners (Mori *et al.,* 2006), could act as modulators of the immune system, and the net result might largely depend on the dosage, the congener(s), and the species in question. This has been observed for phagocytosis in studies that focused on various pinniped species (Levin et al., 2005) and cetaceans (Levin *et al.,* 2004). Specifically, Steller sea lion, *Eumetopias jubatus*, neutrophils increased phagocytosis when exposed to a combination of dioxin + dioxin-like PCBs, although the other non dioxin-like congeners + dioxin combinations reduced phagocytosis (Levin *et al.,* 2005). This is why for pinnipeds, the toxic equivalent factors (TEF) calculated for humans and wildlife (Van den Berg *et al.,* 1998) are not good predictors of PCB effects (Mori *et al.,* 2006; Levin *et al.,* 2004; 2005b).

From an environmental perspective, PCB-induced immunomodulation could be of concern as it could drive the immune response to an anergic state in which the exposed cells cannot implement a full response when challenged by a given pathogen, as they have been incompletely stimulated in the past (Mori *et al.,* 2006). There is some evidence of this in other species. For example, low doses of pesticide can induce a long-term immunosuppression in frogs although it has unpredictable immune effects in the short term (Albert *et al*., 2007). Furthermore, rats affected in a perinatal stage by pesticides and dioxin-like related compounds are more vulnerable to the toxic effects, and this can also be sustained for pinnipeds (Ross *et al*., 1996).

The non dioxin-like congeners considered in this work are frequently measured in free-ranging marine mammals (Del Toro *et al.,* 2006; Niño-Torres *et al.,* 2009, 2010; Ylitalo *et al.,* 2005) and some of them (i.e. PCB 180) are persistent in the environment due to their highly

chlorinated structure (Beyer and Biziuk, 2009). Furthermore, if sea lions are being exposed to pesticides and dioxin-like related compounds during the perinatal stage, it is possible that they are more susceptible to the immunosuppressive effects, as has been suggested for rats (Smialowicz *et al.,* 2001; Smialowicz, 2002) and phocids fed on a diet of polluted herring (Ross *et al.,* 1996b).

To conclude, this study has shown that cryopreserved cells extracted from CSL lymph nodes collected from fresh carcasses display innate NK cell-like activity, and that cells remain viable for at least one month after collection. Furthermore, this study presents a model for future toxicology assays of free-ranging dead animals for which collection of blood samples is often not feasible owing to the quick loss of viability of circulating mononuclear cells.

In general, dioxin-like and non dioxin-like PCB congeners exerted different effects on CSL LNMC. Dioxin-like congeners seemed to have a less pronounced effect on proliferation compared to non dioxin-like congeners, similar to what has been reported for other immune effectors of various marine mammals. These congeners had a more noticeable effect at lower concentrations than at higher concentrations, where the effects tended to disappear. However, in terms of NK cell-like activity, only congener 169 was shown to impact CSL LNMC. Non dioxin-like congeners 153 and 138 had the opposite effect, with a concentration-dependent pattern.

Based on a search of the published literature, it appears that this is the first time that *in vitro* suppression of NK cell activity by a dioxin-like congener has been observed in a pinniped, and is also the first time that a non dioxin-like congener has shown an immunosuppressive effect on *in vitro* lymphocyte proliferation in an otariid pinniped, as was recently reported for a phocid pinniped. The results reported in this study could constitute evidence that the immune system of

free-ranging CSL could become modulated by PCBs, making it difficult to respond to challenges, including oncogenic viruses and cell transformation. Further studies should aim to test whether the *in vitro* results herein obtained are observable at the organism level in natural populations.

Finally, PCB congeners are commonly found as mixtures in the environment (Robertson and Hansen, 2015) and their effects could increase or decrease by antagonistic or synergistic interactions. As the Toxic Equivalence Factor is not predictive for CSL, future studies will need to test various common environmental mixtures to examine differences between effects caused by exposure to individual congeners and those exerted by mixed congeners.

6. SPATIAL AND AGE DEPENDENT VARIATION IN THE TRANSCRIPTION OF IMMUNE-RELATED GENES OF CALIFORNIA SEA LIONS

6.1 Introduction

The California sea lion (hereafter, CSL) inhabits from the central Western coast of Mexico to British Columbia, Canada (Bigg, 1985). The CSL is affected by urogenital metastatic carcinoma, a pathology that is currently the cause of mortality of 25% of individuals stranded along the Californian coast (USA) that were subjected to *post-mortem* analysis (Browning *et al*., 2015). Urogenital carcinoma appears to have an heterogeneous aetiology, as it has been statistically related to different factors such as inbreeding (Acevedo-Whitehouse *et al*., 2003a), individual genetic conformations at specific genes (i.e. heparanase 2, Browning and Acevedo-Whitehouse *et al*., 2014; and the MHC class II DRB loci, Bowen *et al*., 2005), presence of beta-haemolytic streptococci (Lipscomb *et al*., 2000), infection by OtHV-1, a potentially oncogenic herpesvirus (Lipscomb *et al*., 2000; Buckles *et al*., 2006) and high concentrations of environmental organic pollutants, especially DDT and PCB congeners (Ylitalo *et al*., 2005). An unknown combination of all these factors, and most likely others as yet unknown, is likely to be the cause of the increasing incidence of this pathology.

To date, this type of cancer has not been reported in CSL from Mexican waters. However, there does not appear to be a marked difference in the prevalence of OtHV-1 between CSL from the California coastline and those from the Gulf of California (Barragán-Vargas *et al*., 2016), nor

are there evident differences in levels of inbreeding and individual genetic conformation of the MHC class II between both regions (Bowen *et al*., 2006; Montano-Frías *et al*., 2016). The only known difference in risk factors between regions appears to be the levels of organochlorine contaminants, which are two to three levels of magnitude lower in CSL from the Gulf of California than in individuals from California (see Ylitalo *et al*., 2005; Niño-Torres *et al*., 2009).

Interestingly, despite the lack of evidence of cases of urogenital carcinoma, cellular transformation of the genital epithelium has been observed in CSL from some reproductive colonies in the Gulf of California (Barragán-Vargas *et al*., 2016). Studies with humans and laboratory animals have shown that cellular transformation is the first step to develop cancer (Chaffer and Weinberg, 2015). Considering that most of the risk factors associated with urogenital carcinoma are present in the Gulf of California, it is likely that occurrence of cancer is not only related to the presence of oncogenic pathogens and contaminants but to their relative concentration and the intensity of exposure. In turn, it is likely that the outcome of cell transformation will be associated to the competence of the CSL immune responses, particularly to those responses that are related to the risk factors identified to date, as well as others not yet known. If pro-oncogenic factors vary geographically, immune effectors related to viral and oncogenic vigilance would expectedly show different patterns among regions, and could help explain why some CSL populations exhibit cellular transformation of the genital tract while others do not (Barragán-Vargas *et al*., 2016).

Some studies have analysed the CSL population along the Gulf of California according to different criteria for regionalization. Population trends define four regions in the Gulf of California (Ward *et al*., 2010), namely the northern region (which contains three CSL colonies: Rocas Consag, Lobos, and San Jorge), North-central region (with three colonies: Granito, Cantiles, and

Los Machos), South-central region (with five colonies: Partido, Rasito, San Esteban, San Pedro Mártir, and San Pedro Nolasco) and the far South region (that includes two colonies: Los Islotes and Farallón de San Ignacio). Mitochondrial sequences also define four genetic clusters within the Gulf of California (Maldonado *et al*., 1995) although geographical characterization was not considered by the authors and, thus, no inferences of regions were obtained. A wider, more recent, study described three genetic clusters within the Gulf based on molecular differences in region I of the mitochondrial control region (Schramm *et al*., 2009). These clusters defined the northern colonies (Lobos, Granito, and San Jorge), central colonies (San Esteban and Cantiles) and southern colonies (Los Islotes) (Table 6.1).

Table 6.1. CSL regions in Gulf of California previously defined by different criteria. N.A. indicatess that this colony was not included in the study.

	Population trends	Mitochondrial differentiation
Rocas Consag	1	N.A.
Lobos	1	1
San Jorge	1	1
Granitos	2	1
Cantiles	2	2
Los Machos	2	N.A.
Partido	3	N.A.
Rasito	3	N.A.
San Esteban	3	2
San Pedro Mártir	3	N.A.
San Pedro Nolasco	3	N.A.
Los Islotes	4	3
Benitos	N.A.	N.A.

Additionally, oceanographic and bathymetric variables can influence nutrient availability and pollutant concentration or redistribution due to marine currents and zones of upwelling (Santamaría-del-Angel *et al*., 1994). Some of these factors, important for most marine predators (Grémillet *et al*., 2008), can affect the behaviour and physiology of individuals differentially between sexes (González-Suárez et al., 2009). For instance, due to their life history, female adults remain close to the colony during the entire year so they do not follow the marine currents, and are more affected by local circumstances such as minimum oxygen zones and changes of local resources (Aurioles-Gamboa *et al*., 2017).

There are also regional differences in terms of pollutants, which follow a latitudinal pattern. The northern region is more polluted than the South due to sustained spillover from the Colorado River delta (García-Hernández *et al*., 2001; 2006). In addition, the interchange of water is biased due to the distribution of the currents. In the midriff, the southern and northern currents turn, not allowing a full admixture of the water (Santamaría-del-Angel *et al*., 1994). In addition, other areas, such as the Sinaloa coastline in the south-eastern Gulf of California, where the colony Farallón de San Ignacio is found (Galindo-Reyes *et al*., 1999), shows evidence of recent organochlorine pollution (Reyes *et al*., 1999).

Thoroughly fragmenting the Gulf of California based on many different oceanographic variables (Santamaría-del-Angel *et al*., 1994) would be inadequate to understand ecological differences in the CSL population. However, some of the variables that differ among regions could be relevant. Islands located near the land have different oceanographic conditions than those in the central region of the Gulf. For instance, phytoplankton profiles and upwellings cary among

different areas (Round, 1967; Lluch-Cota, 2000). Due to that, to assume an *a priori* grouping of CSL colonies could limit our understanding of ecological patterns of the CSL population.

Based on the reported genetic differences described above, and the variation in oceanographic and ecological factors, it is likely that CSL experience intra- and interregional differences as well as inter-colony differences in their exposure to various factors, such as prey (Santamaría-del-Angel *et al*., 1994; Aurioles-Gamboa *et al*., 2017) and pathogens (Acevedo-Whitehouse et al., 2003b; Godínez et al. 1999) that could impact their immune system. Prey is the source of nutrients and metabolic energy for sea lions. As its availability is determined by the productivity of a region, (Aurioles-Gamboa *et al*., 2017), CSL cannot invest resources in their immunity independently of their environment (Vera-Massieu *et al*., 2015). In the closely-related Galapagos sea lion, *Zalophus wollebaecki*, these trade-offs and limitations have been described, and have been related to anthropogenic influence (Brock *et al*., 2013).

In addition to potential differences in exposure to different extrinsic factors, ontogenetic differences could influence the immune system. The immune system of young mammals is often immature to develop a fully adequate response and antigenic stimulation is needed before the immune system works correctly (Durandy, 2003). For instance, the adaptive immune system of children develops early, but it is inefficient for long periods because of its naivety. Moreover, innate cytotoxicity and phagocytosis hardly work until maturity (Durandy, 2003). However, pinnipeds appear to mature quite early (King *et al*., 1998; Espinosa-de Aquino *et al*., 2017), most likely due to strong selective pressure during their early development. The immune system of adult CSL females, on the other hand, is fully mature but, at the same time, has been exposed to many challenges throughout their lives, including immune-suppressive agents that could impair its proper function (Cohn *et al*., 2007; Cole *et al*., 2012). Furthermore, pregnant or lactating females

are likely to downregulate various immune effectors (Gill, 1985; Ross *et al*., 1993; Raghupathy, 2001) as a means to avoid immune pathology towards the developing fetus.

As some of the regions where CSL breed within the Gulf of California are considered to be relatively polluted due to industrialization and agriculture spillover (Álvarez-Romero *et al*., 2013), and organic pollutants have been related to cancer (Ylitalo *et al*., 2005), I hypothesized that transcription of key immune-related genes is likely to vary spatially, and group according to ecological characteristics. I also considered that due to ontogenetic constraints, there would be differences between the relative transcription levels between pups and adult female CSL.

6.2 Methods

6.2.1 Sample collection

Samples of peripheral blood were collected from 130 two-month old pups and from 52 adult female CSLs. All animals were captured at breeding colonies within the Gulf of California and the Mexican northern Pacific (see Table 6.1). The colonies are located on islands that have different levels of human influence as well as ecological differences (see introduction). Blood (7-10 mL) was extracted from the caudal gluteal vein of the physically-restrained individuals, and was preserved in sodium heparin Vacutainer vacuum tubes (Neubert *et al*., 1991). Heparin-preserved blood was centrifuged immediately after collection at 3200 rpm for 10 min on a clinical centrifuge in order to separate the buffy coat, which was stored immediately in cryogenic tubes and frozen in

liquid nitrogen. Back in the laboratory all samples were transferred to a -°80 C freezer where they remained until processing.

6.3.2 Relative quantification of gene expression in lymphoid subpopulations

The buffy coat samples were used to extract total RNA using Trizol (Sigma-Aldrich, USA) as per the manufacturer's instructions. RNA integrity and quantity were assessed by gel visualization and by spectrophotometry (Nanodrop, Qiagen). Good quality samples were considered as those whose A_{260}/A_{280} ratio fell between 1.75 and 2.10 (Fleige and Pfaffl, 2006). Reverse transcription was performed using 200ng of RNA in 14 uL of reaction following the manufacturer's instructions (QuantiTect Reverse Transcription Kit, Qiagen).

Quantitative PCR was conducted to measure the relative expression of specific genes characteristic of lymphocyte subpopulations. KIR, Ly49, perforin, and granzyme B genes were examined to assess NK cell function. These genes were selected because KIR and Ly49 transcripts are known to be present and functional in the California sea lion (Parham, 2008; Hammond et al., 2009) and are expressed once NK cells reach maturity. Furthermore, perforin and granzyme B are expression of markers of cytotoxic activity (Trapani *et al*., 2002; Trapani and Sutton, 2003), and, such as, are essential for antitumoral activity.

To assess the activity of T-lymphocyte subpopulations, the relative expression of EOMES, which is considered an activation marker for $TCD8^+$ (Pearce *et al*., 2003), STAT1, a transcription factor (Zheng and Favell, 1997), and Tbet, a master regulator of differentiation (Dorfman *et al.*, 2003) were quantified. These genes were selected as Th1 markers for $TCD4^+$, while Th2 transcripts

selected were GATA3, a master regulator of differentiation, and STAT6, a transcription factor (Zheng and Favell, 1997). Treg activity was assessed by FoxP3 transcription levels (Hori *et al*., 2003).

RPS5 and HpRT genes were used as control genes for the SYBR-green qPCR assays, as they are expressed in all nucleated cells and were previously standardized in our lab. Furthermore, they performed adequately at the annealing temperature ranges for the target genes and were good early- and late-expression markers. This means that one of the reference genes was expressed along with some targets (in a -5, +5 cycle range) and the other gene began to be measured around 10 steps later, with the rest of the targets in the same range (-5, +5 cycles). Table 6.2 shows the selected genes and the main cell type that expresses these genes. E and R^2 results for all the primers for qPCR can be seen in Appendix 1, together with details on standarization. The threshold was determined visually between parallel lines, after the amplification take-off.

Reaction mixes contained 4 µL of cDNA (a 1:4 to 1:16 dilution of template previously generated by retrotranscribing 10 ng/µL of RNA; see Chapter IV), 0.15 µL of each primer (at 0.2µM each), 5 µL SYBR® Green master mix (Thermo Fisher), and 0.7 µL of water to reach 10 µL of final volume. Quantitative PCR was run as follows: 95ºC for fifteen minutes, followed by 40 cycles corresponding to 15 seconds at 95º, one minute at 55ºc (during which the plate was read) and 72ºC for 1 minute. The reaction was maintained at 95ºC for 15 seconds. The melting curve was read every 15 seconds with 0.5 ºC increments between 60 and 90ºC.

When the amplification reactions were run at 55ºC (TM) all primers showed an estimation index between 90 and 110% and were adjusted over 0.965 (R^2>0.965). The data were considered reliable if the difference between replicates was below one cycle. If reference genes reliability failed, all samples were repeated. Optimal results were achieved when using 1:2 to 1:8 of cDNA.

Table 6.2. Genes used for relative quantitation of transcription levels in leukocyte cDNA.

Gene	Role	Mainly expressed in:
Ly49	Target gene	NK cells (Hammond et al. 2009)
Tbet	Target gene	Th1 T cells (Dorfman et al. 2003)
FoxP3	Target gene	T reg (Hori et al. 2003)
GATA3	Target gene	Th2 T cells (Zheng and Flavell 1997)
STAT-1	Target gene	Th1 T cells (Zheng and Flavell 1997)
KIR	Target gene	NK cells (Hammond et al. 2009)
EOMES	Target gene	Cytotoxic T cells ((Pearce et al. 2003))
Perforin	Target gene	Cytotoxic T cells and NK (Fehniger et al. 2007)
GranzymeB	Target gene	Cytotoxic T cells and NK (Fehniger et al. 2007)
STAT-6	Target gene	Th2 T cells (Zheng y Favell, 1997)
HPRT	Internal control gene	All nucleated cells
RPS5	Internal control gene	All nucleated cells

6.2.3 Statistical analyses

All statistical analyses were conducted using R v3.4.2 (R Core Team, 2017) and R-Studio (RStudio, 2015). Normality and homoscedasticity of all the response variables was tested with a Shapiro test and a Barlett's test, respectively. In addition, the distribution of the residuals was examined using Q-Q plots, Cook distance and ploting the adjusted residuals against the obtained residuals.

For explanatory variables that did not show evidence of deviations of normality and homoscedasticity, a general linearized model (GLM) was constructed for each comparison instead of using ANOVA, as the numbers of samples in each group differed greatly. Explanatory variables that did not pass the normality test were further investigated with Cullen and Frey graphs to determine the distribution of the error and select an adequate family for the GLM. Explanatory variables used were colony and age, as well as their interaction.

In order to analyse gene transcription levels using no pre-defined regionalization, I determined natural clustering of the gene transcription data recorded for pups and adult CSL by using Ward hierarchical and average cluster analysis (Johnson, 1967), and the different models were compared in order to select the one with the better fit. As there were many data points for which transcription results were not of sufficient quality to be used, a multiple imputation chain equation (MICE), which can be used when data are missing at random (Rubin, 1996), was applied. The imputed values were generated using *mice* package in R (Buuren, 2010) with the default transformation (Euclidean).

Next, heat maps and dendrograms were created using different gene combinations and algorithms. Heat maps were drawn using Euclidean metrics and the complete method. Three different dendrograms were created, and the one that reported a highest cophenetic correlation for each (i.e. equation which calculates the similarity between cophenetic distance and the distance for the algorithm used for the dendrogram) was selected. The algorithms used were Ward.D2 (an R update of Ward), Average and Euclidean; the latter being used when the other algorithms were uninformative.

Initially the entire set of target genes was used to find if there were relevant groups considering all genes as the “immune response” of an individual. Then, clustering of gene

expression was examined using a smaller number of genes representative of each kind of response. Th1 response genes (STAT-1, Tbet), Th2 response genes (GATA3, STAT-6), CD4 (which included Th1 and Th2 genes), CD8 cytotoxicity related genes (Eomes, perforin and granzymeB), NK related genes (Ly49, KIR, perforin and GranzymeB), and Treg genes (FoxP3) were used to create independent dendrograms for pups and adults. For the cluster analysis, the number of natural clusters was determined according to a rarefaction curve using within sum of squares criterium to establish which number of groups had sufficient explanatory power and discard those that fragmented the sample into too many non-informative groups. Rarefaction curves were built with *factoextra* package (Kassambara and Mundt, 2016). The number of groups was also tested using the *cluster* package (Maechler *et al*., 2012) in R, and the cluster.stats function was used to identify which group was most informative among those selected.

6.3 Results

Data differed in reliability among genes, as some began to take off in the last steps of the qPCR. While qPCR for STAT-1, perforin, granzymeB, Tbet, Ly49, GATA3, Eomes and FoxP3 reported more than 140 reliable data, STAT-6 and KIR were both below 50 reliable data. Relative transcription of STAT-1 ($p=0.053$), perforin ($p=0.553$), granzymeB ($p=0.108$), Tbet ($p=0.525$), KIR ($p=0.363$), Ly49 ($p=0.099$), GATA3 ($p=0.419$) and FoxP3 ($p=0.199$) showed no evidence of deviation from normality with the Shapiro-Wilk test. When testing for homocedasticity, neither KIR ($p= 0.004$) nor STAT-1 ($p=0.017$) passed Bartlett's test. Eomes ($p=0.002$) and Stat-6 (0.021) deviated significantly from expectations of normality and, as was the case for KIR and STAT-1,

were transformed to fit a 0 to 1 distribution of data and a GLM based on their quasibinomial distribution.

STAT-1

Relative transcription levels of STAT-1 varied among colonies (GLM; $F_{174,12}$=3.11, p=0.0001), with the lowest expression observed for CSL from Los Machos (p=0.017) and Granito (p=0.014). Age also played a role in this model (GLM; $F_{174,1}$=69.01, p=5.543x10^{-14}), with expression being higher for pups than for adults in Lobos, Granitos, Machos, Rocas Consag, and San Jorge colonies (GLM; $F_{174,12}$=2.73, p=0.002; Fig. 6.1).

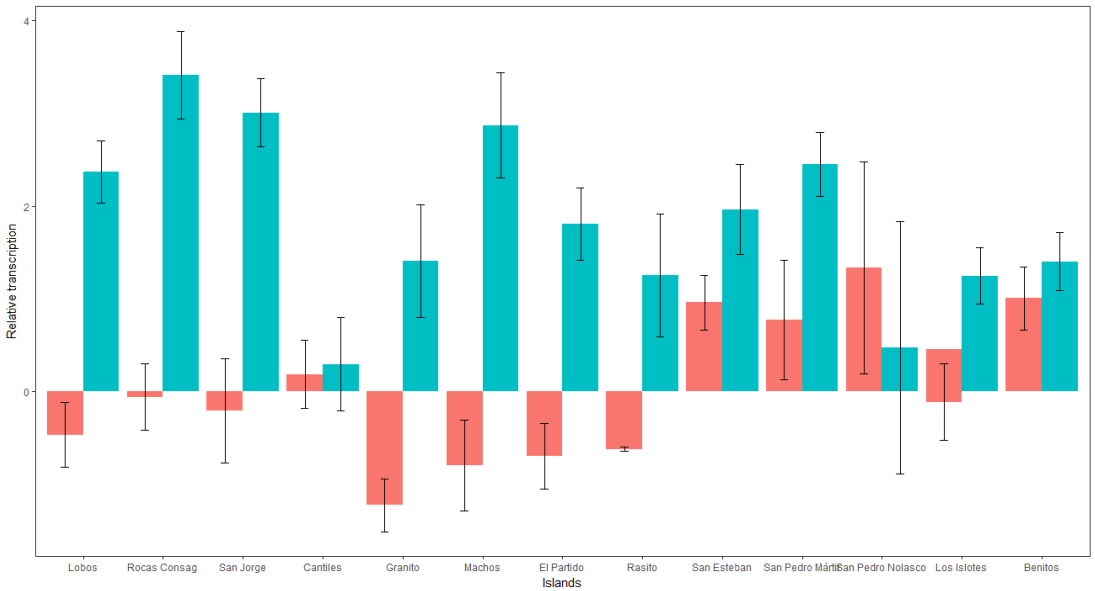

Figure 6.1 Relative transcription of STAT-1 in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

STAT-6

Relative transcription levels of STAT-6 were difficult to analyse in the same way as the other genes due to the low number of samples (24) that were of sufficient quality based on the previously established criteria. Furthermore, it was impossible to compare expression levels by age, as robust data was available only for pups from four colonies. With the data available, relative expression levels did not vary among colonies (GLM; $F_{23,3}$=1.639, p=0.058; Fig. 6.2).

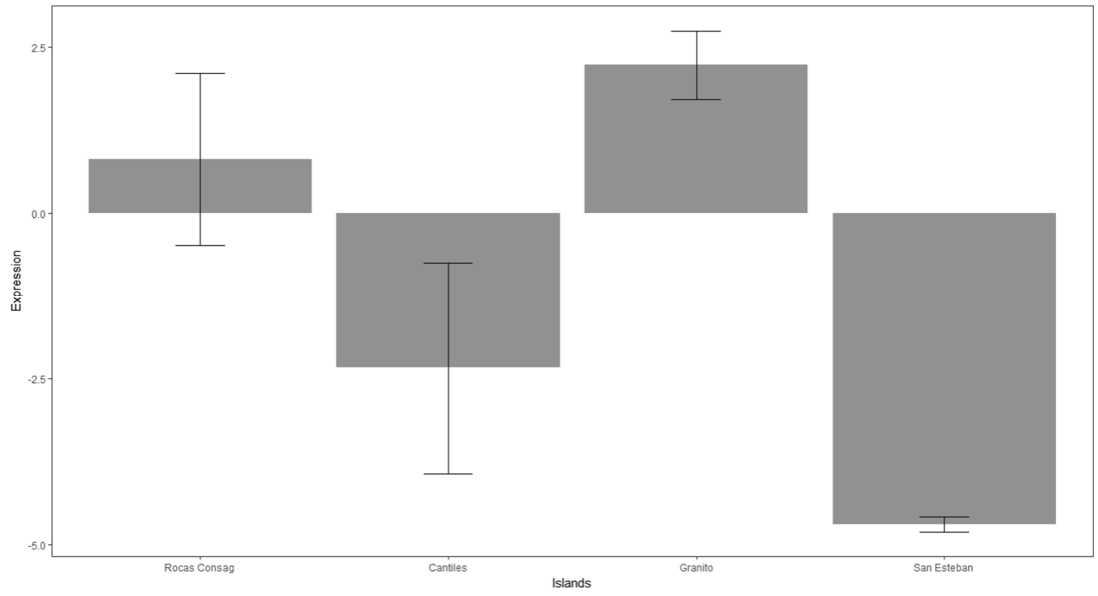

Figure 6.2 Expression of STAT-6 in circulating leukocytes of CSL pups from different islands. Bars represent the standard error of the mean.

GATA-3

Relative transcription of GATA-3 varied significantly among colonies (GLM; $F_{174,12}$=3.179, p=0.001; Fig. 6.3), with lower transcription levels observed in CSL from Cantiles (p=0.047), El

Partido ($p=1.34x10^{-5}$), Granito ($p=0.027$), Los Machos ($p=0.002$), and Rocas Consag ($p=0.041$). Pups had higher expression levels than adult females in all colonies (GLM; $F_{174,1}=61.251$, $p=8.59x10^{-13}$), and the interaction between age class and colony was significant in the model (GLM; $F_{174,12}=2.351$, $p=0.009$).

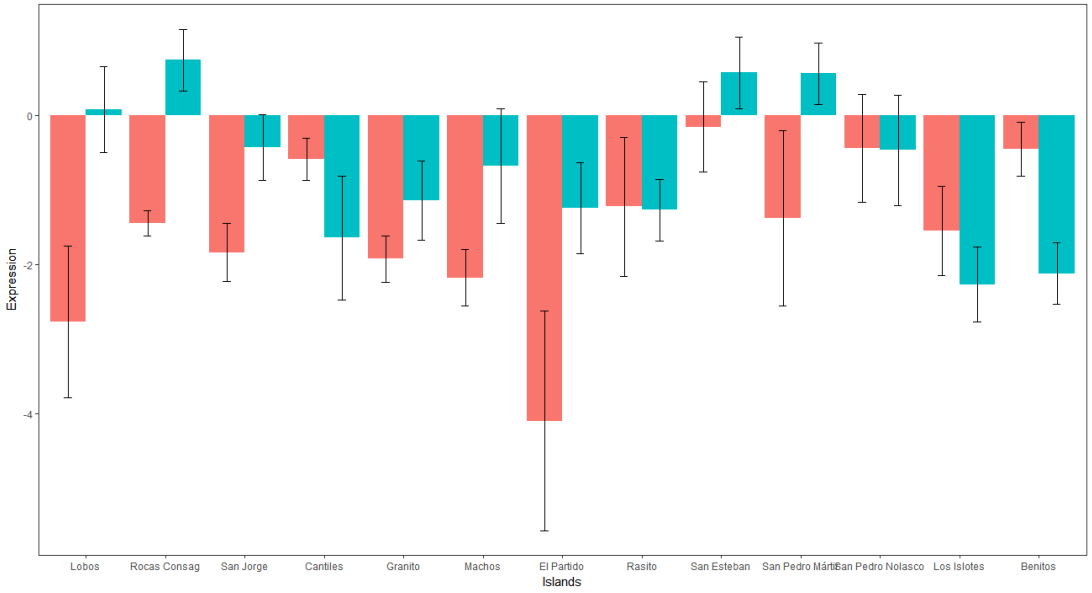

Figure 6.3 Expression of GATA3 in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

KIR

As occurred for STAT 6, the analysis of relative transcription levels of KIR was also limited due to the low number of samples with sufficient quality, which were: 7 (Cantiles), 4 (Granito), 8 (Rocas Consag) and 2 (San Esteban). However, there were differences among colonies (GLM; $F_{20,3}=3.998$, $p= 0.025$) with the highest level of transcription observed for sea lions from Rocas Consag ($p=0.014$; Fig. 6.4).

Ly49

Relative expression of Ly49 varied among colonies (GLM; $F_{175,12}$=3.029, p=0.001), being overexpressed in pups from Lobo and in adult females from Cantiles and from Rasito (Fig. 6.5) .

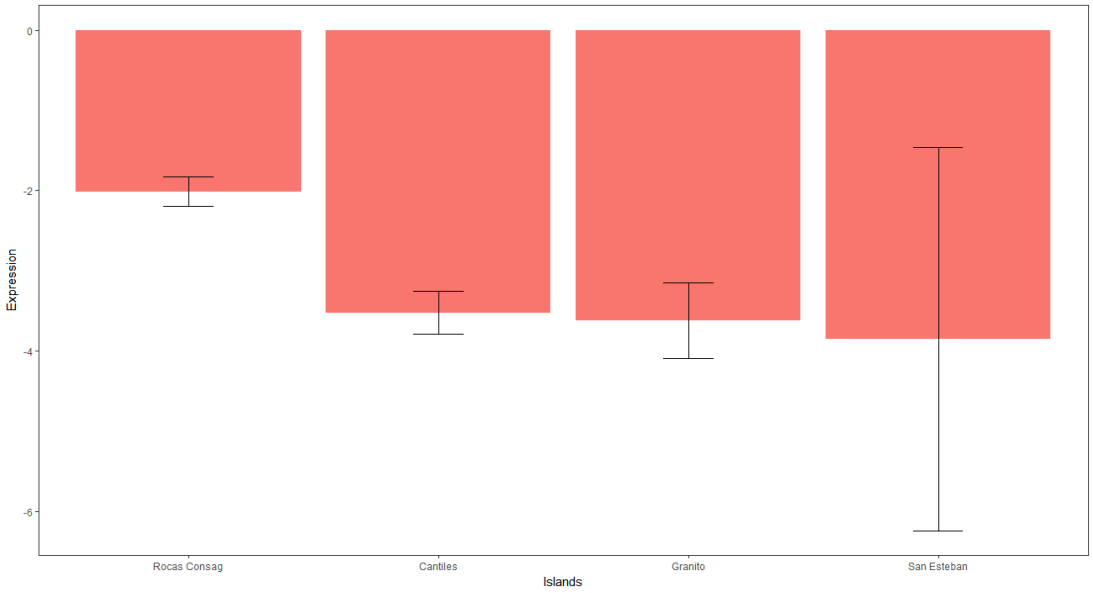

Figure 6.4 Expression of KIR in circulating leukocytes of CSL pups from different islands. Bars represent the standard error of the mean.

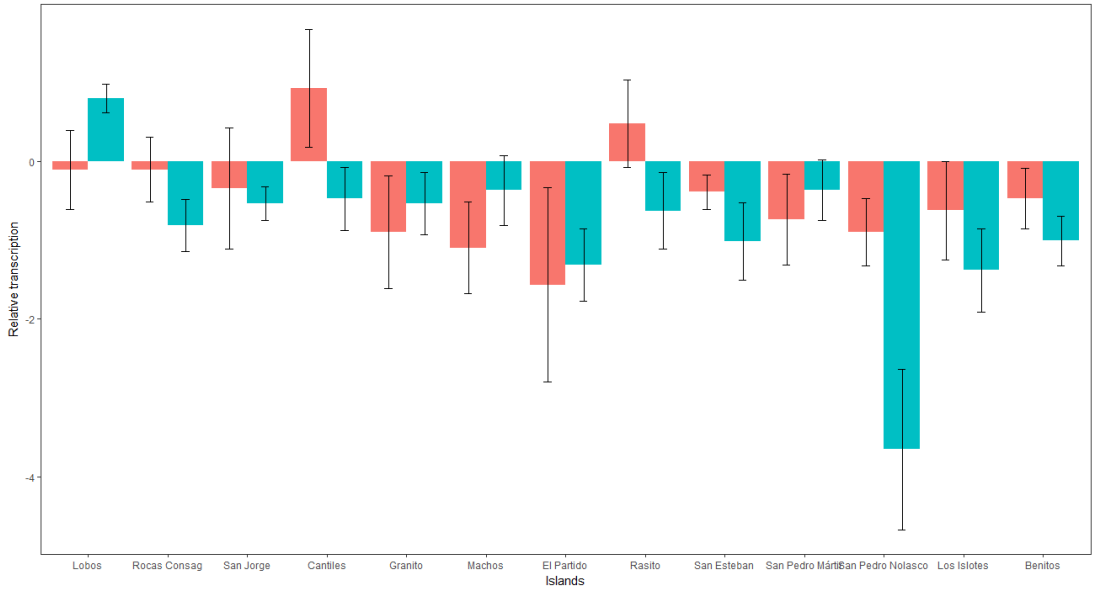

Figure 6.5 Expression of Ly49 in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

Eomes

Eomes transcription levels did not have a normal distribution (Shapiro test; p=0.002). Instead, the data had a quasibinomial distribution. Relative transcription of Eomes did not vary among colonies (GLM; $F_{139,12}$=1.627, p=0.094), although age remained a significant explanatory factor in the model (GLM; $F_{139,1}$=6.264, p=0.014; Fig. 6.6), regardless of its interaction with colony (GLM; $F_{139,11}$=1.197, p=0.297).

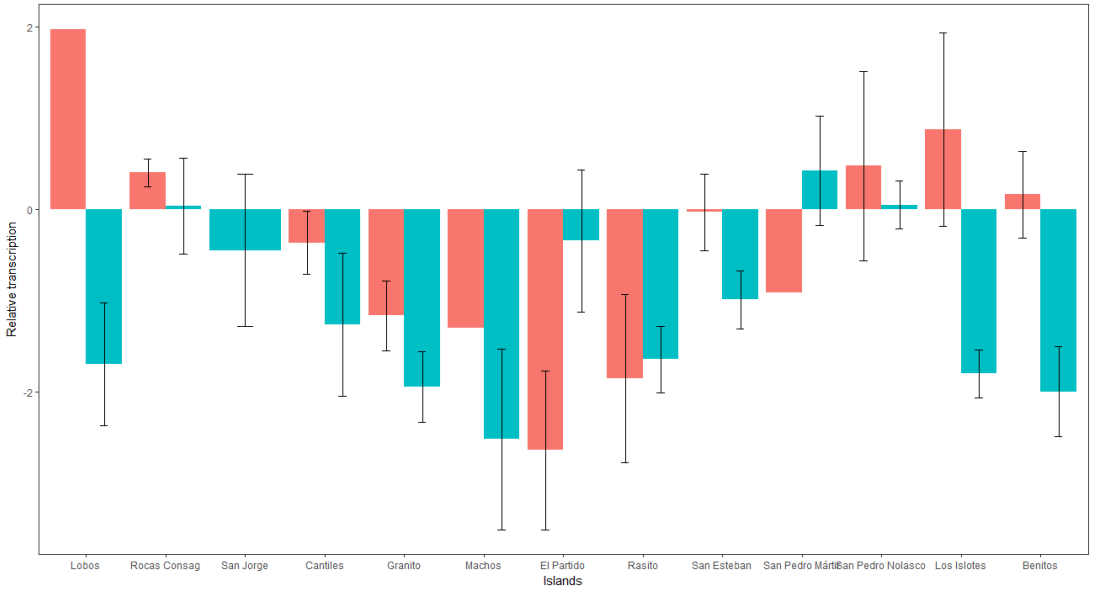

Figure 6.6 Expression of Eomes in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

Tbet

Tbet expression was heterogeneous among colonies (GLM; $F_{182,12}$=2.976, p=0.001), and differences in expression between pups and adult females also varied among colonies (GLM; $F_{182,12}$=2.612, p= 0.003; Fig. 6.7). Adult sea lions from El Partido and Lobos had the lowest transcription levels (p=0.005, p= 0.018, respectively).

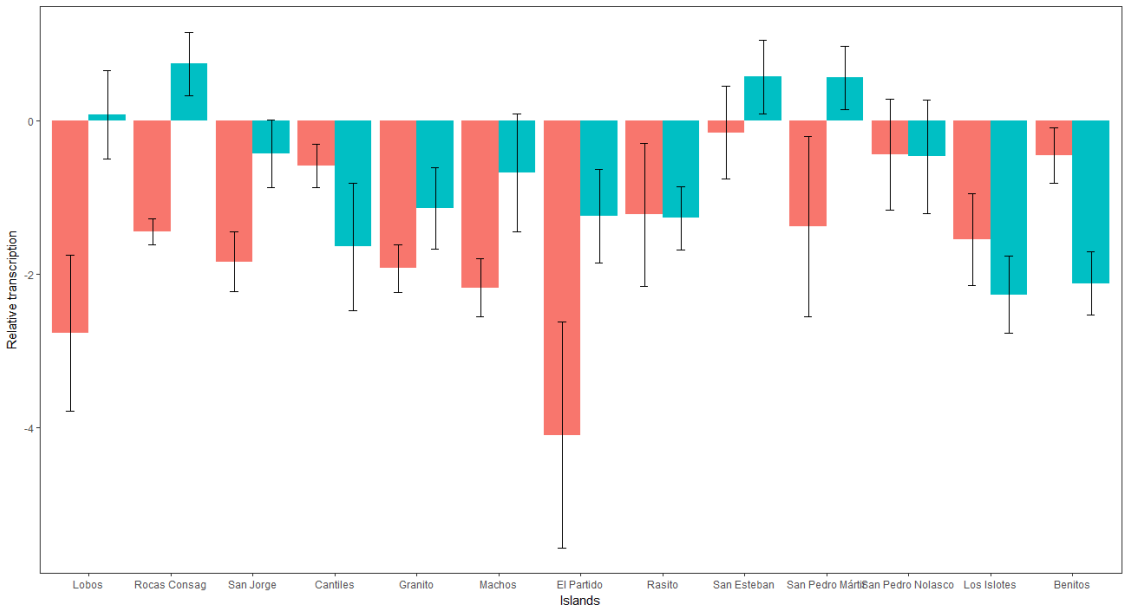

Figure 6.7 Expression of Tbet in in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

Perforin

Relative transcription levels differed among colonies (GLM; $F_{134,12}$=4.774, p=2.981x10^{-6}; Fig. 6.8) and the interaction between colony and age was significant in the model (GLM; $F_{134,11}$=3.277, p=0.001). There were no observable differences in expression levels between pups and adult females (GLM; $F_{134,1}$=2.521, p=0.115).

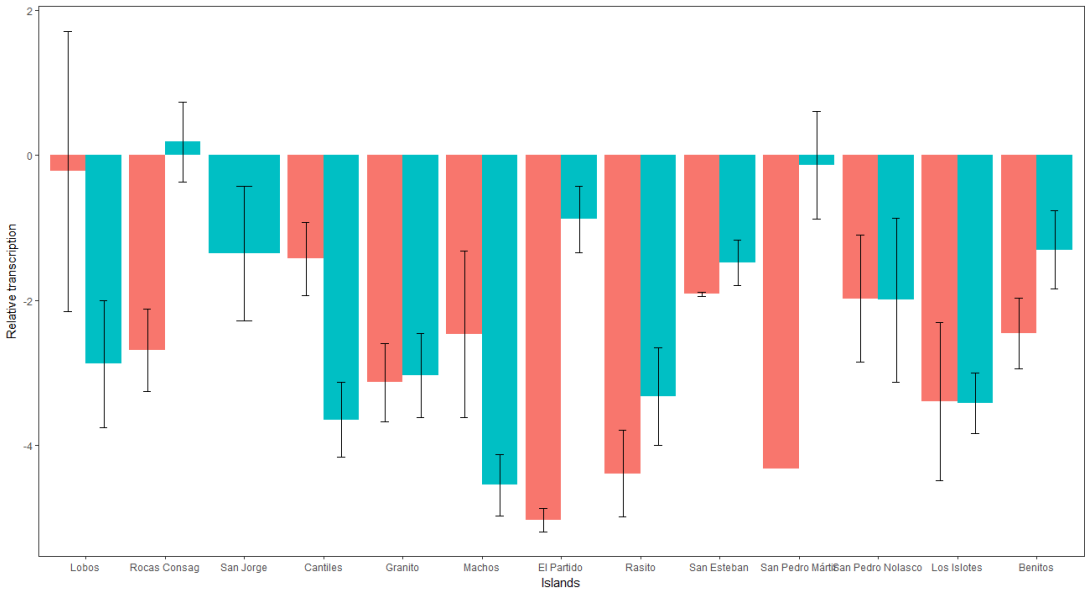

Figure 6.8 Expression of perforin in in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

GranzymeB

GranzymeB transcription levels varied among colonies (GLM; $F_{141,12}$= 3.659, p=0.001; Fig. 6.9), with the highest expression observed for sea lions from San Jorge (p=0.025). There were no differences in transcription levels between pups and adult CSL.

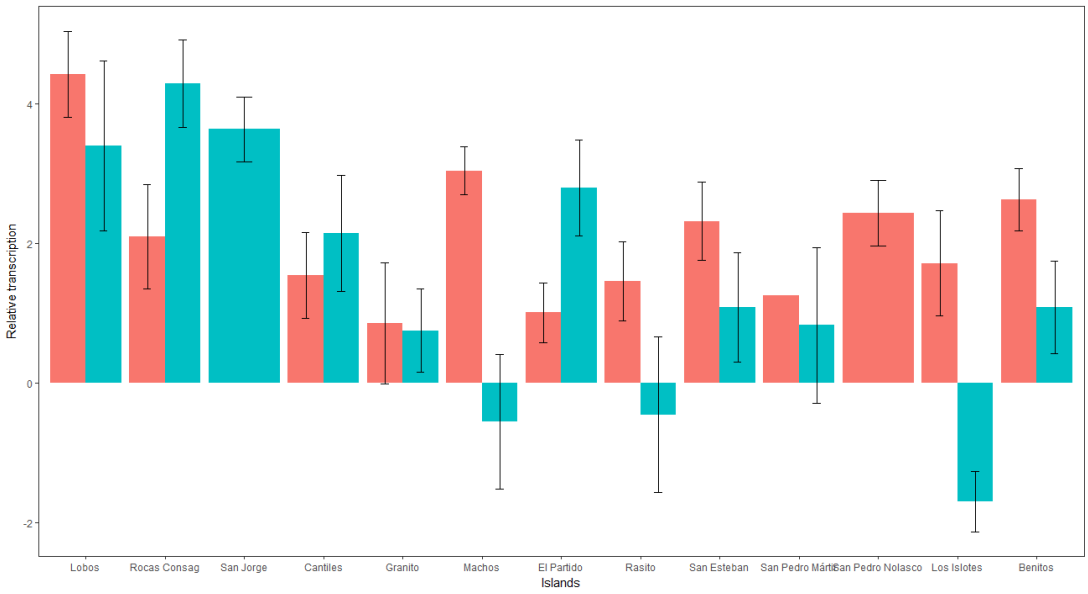

Figure 6.9 Expression of granzymeB in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

FoxP3

Relative transcription of FoxP3 also varied significantly among colonies (GLM; $F_{168,12}$=2.816, p=0.002). Sea lions from El Partido, Granito, and San Jorge showed lower transcription levels (p=0.005, p=0.041, p=0.017) than other colonies. Transcription did not differ between ages and the interaction between colony and age was not significant (GLM; $F_{168,12}$=1.276, p= 0.239; Fig. 6.10).

Analyses of gene transcription levels using no pre-defined regionalization

For the clustering analysis that considered all of the examined genes, correlations were first examined visually. An expression profile was created and a heat map was built using all the genes and all the samples, of both adult and pup CSL (Fig. 6.11). This analysis allows detecting patterns

of interest and identifying the samples within each of the groups. To confirm what is observed in the heat map, I built a rarefaction curve and a dendrogram. Both analyses showed at least three apparent clusters (Fig. 6.11 and 6.12), marked in blue and red in the heat map and as a bend in the rarefaction curve.

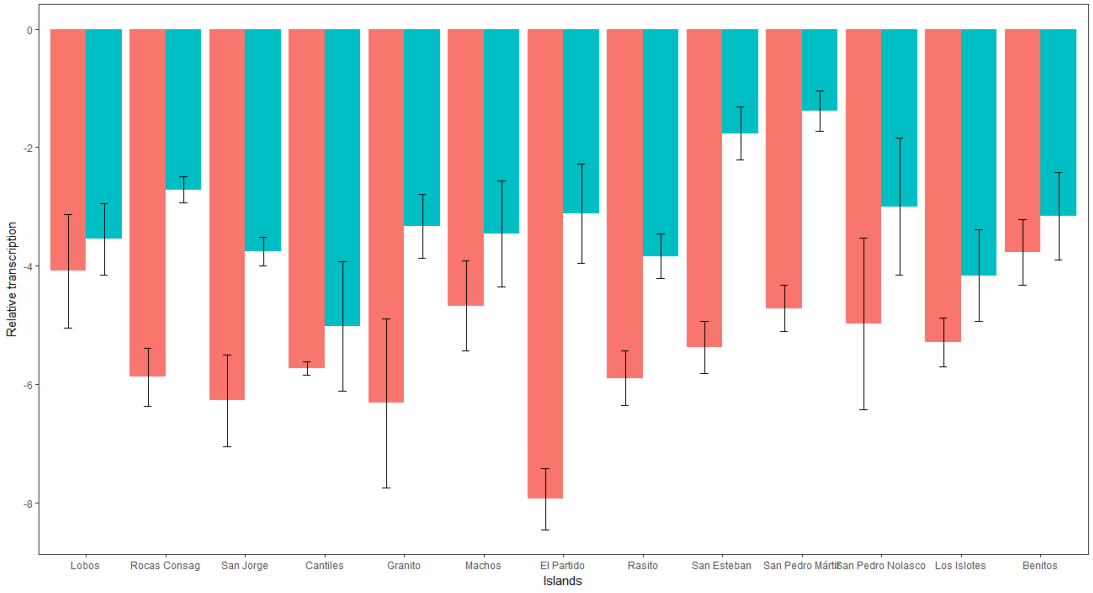

Figure 6.10 Expression of FoxP3 in circulating leukocytes of CSL from different islands. Red=adults, blue=pups. Bars represent the standard error of the mean.

Although groups were formed, the clustering was not explained by geographical or regionalization patterns. This concurs with the reported value for dendrograms built using the ward.D2 algorithm, which was below the optimal cophenetic correlation of 0.75 (0.54), and the average approach (0.65), a slightly better fit according to the cophenetic correlation (Fig. 6.13). The cluster analysis showed the two main components which accounted for 55% of the difference (Fig. 6.14). This was a low resolutive algorithm, as those below 0.75 of cophenetic correlation are inefficient for describing grouping.

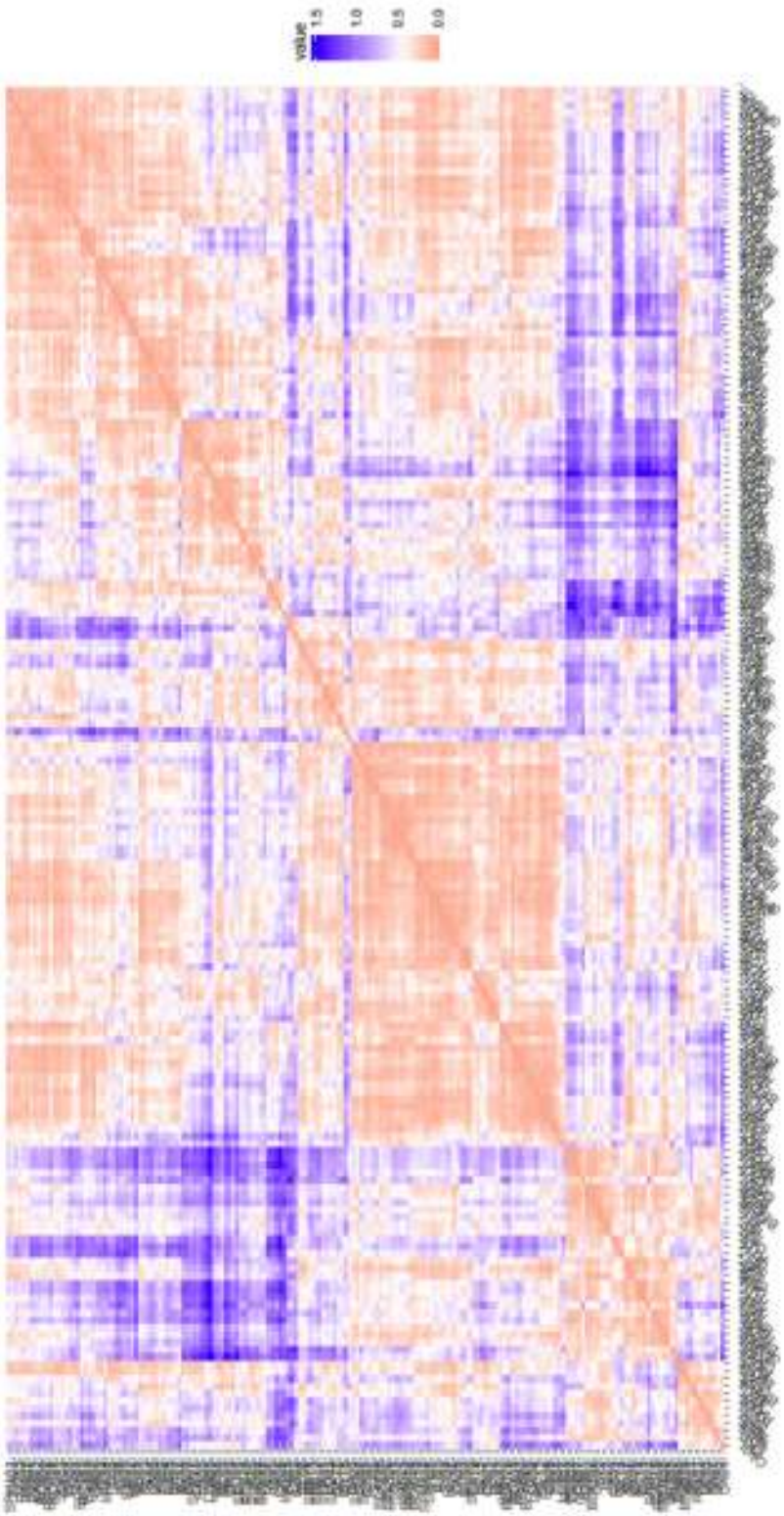

Figure 6.11. Heat map of transcription levels of all immune genes in pups and adult CSL.

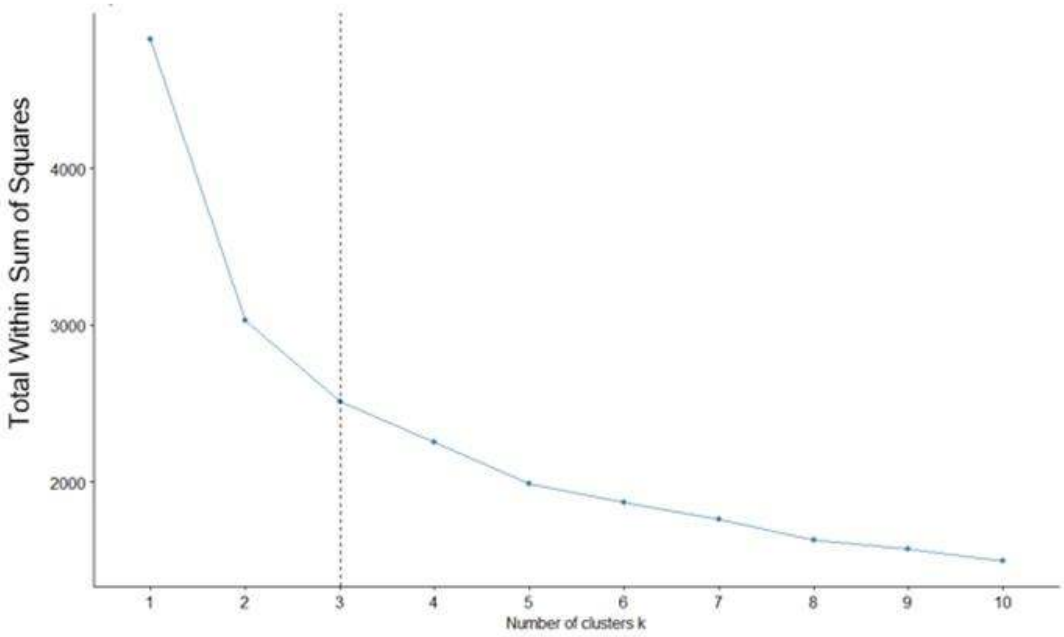

Figure 6.12 Rarefaction curve considering transcription levels of all immune genes analysed for pups and adult CSL. The dotted line shows the optimal number of clusters.

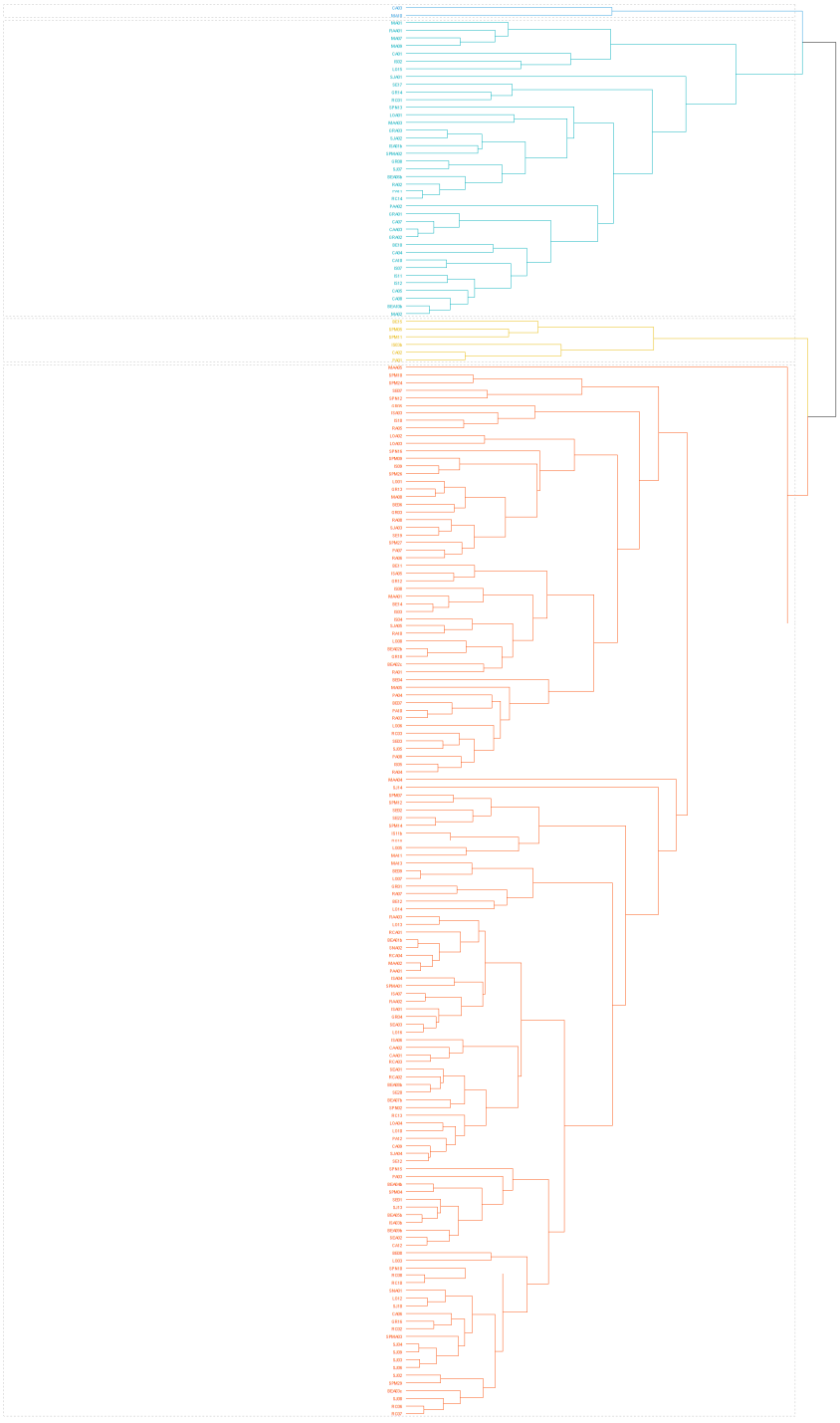

Figure 6.13 Average based dendrogram for transcription levels of all immune genes for pup and adult CSL.

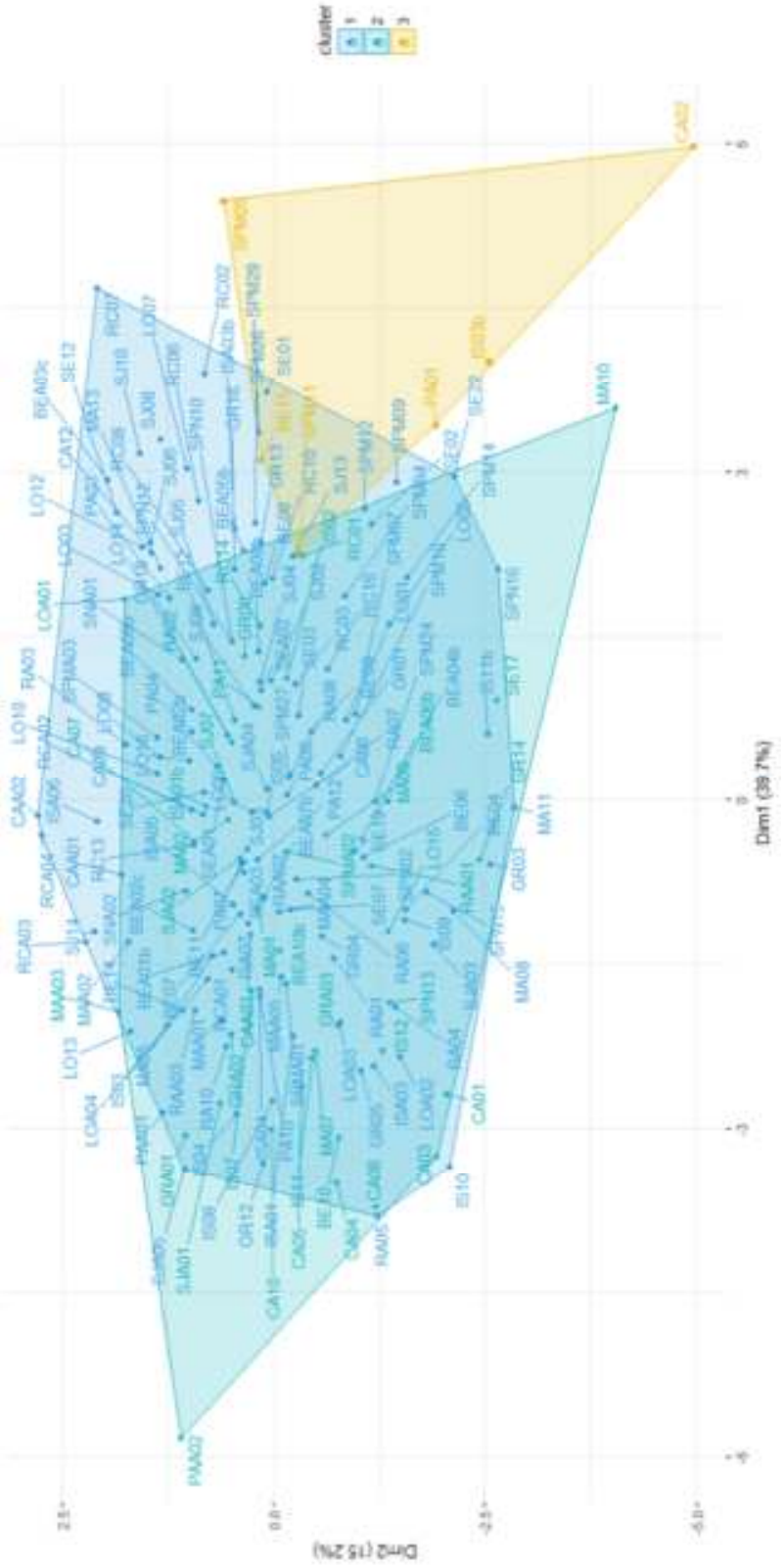

Figure 6.14. Clustering of transcription levels of all immune genes of pup and adult CSL.

Similar results were observed when the cluster was built taking into account all the genes analysed only for CSL pups. The heat map showed a minimum of three large groups (Fig. 6.15), consistent with what was obtained in the rarefaction test. However, the dendrogram using ward.2 and average algorithm provided cophenetic correlation values (0.58 and 0.65, respectively) below what is desired (0.75) of whereas the average one showed a more accurate 0.65 and was represented (Fig. 6.16). However, the cluster plot using the two main components only explained 59% of the variation. Due to that groups were overlapped and showed no pattern (Fig. 6.17).

Gene transcription clustering was more informative when only adult CSL were considered. The heat map showed great homogeneity except for a pair of samples (ISA 03 an LOA02) that appeared to have a very similar behaviour (Fig. 6.18). Six groups were identified in the rarefaction analysis (Fig. 6.19), but three of them included only one animal. This composition of groups with only one animal was explained with the average method dendrogram, whose reported value (0.81) was above the ideal cophenetic correlation (Fig. 6.20). The dendrogram using the algorhythm ward.2 was less informative (0.56). A clear overlap was observed in the cluster analysis, which represents the homogeneity of the samples, but some individuals that behaved diferently were identified; namely ISA03, LOA02 and BEA03c (Fig. 6.21).

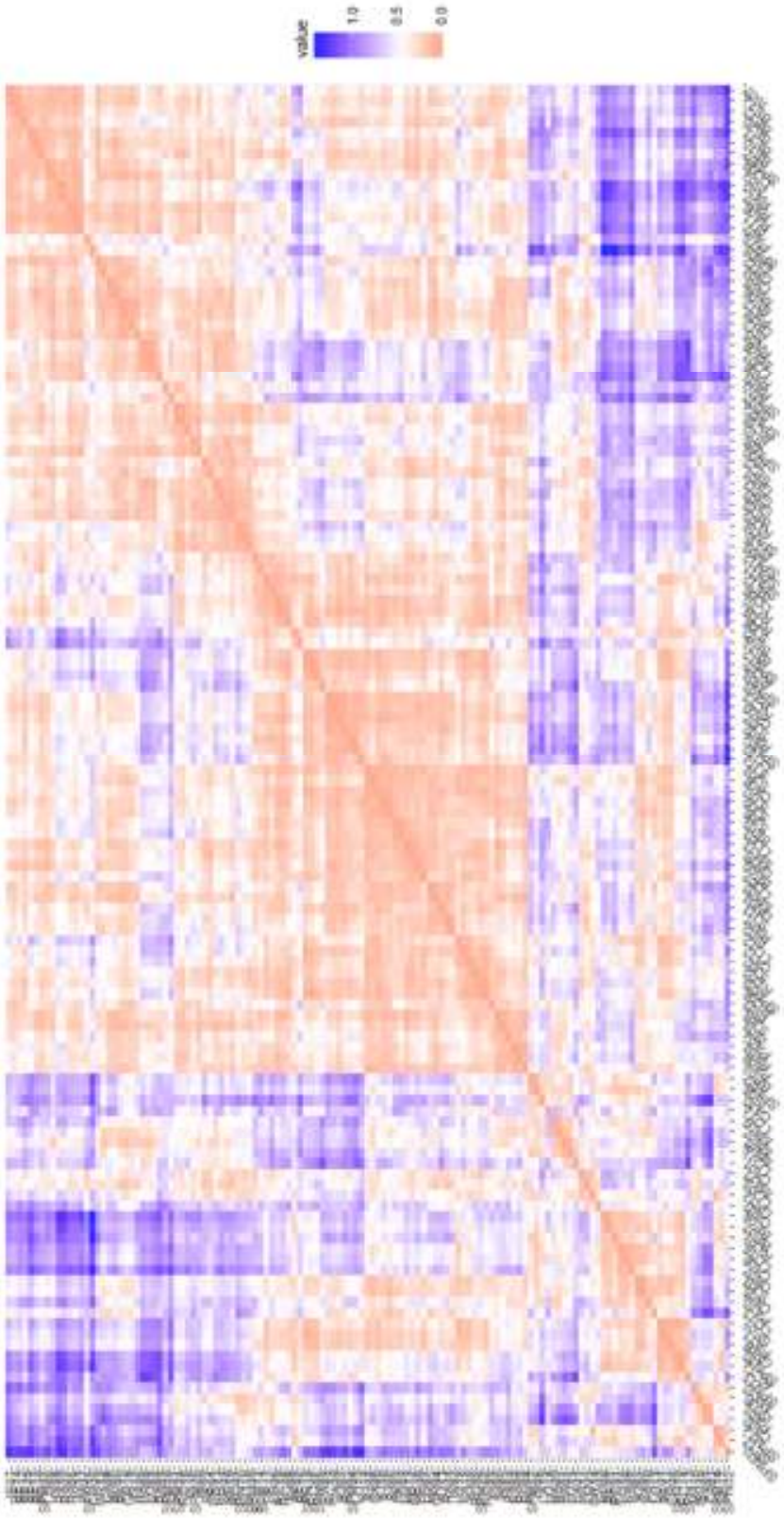

Figure 6.15. Heat map for CSL pup immune gene transcription levels.

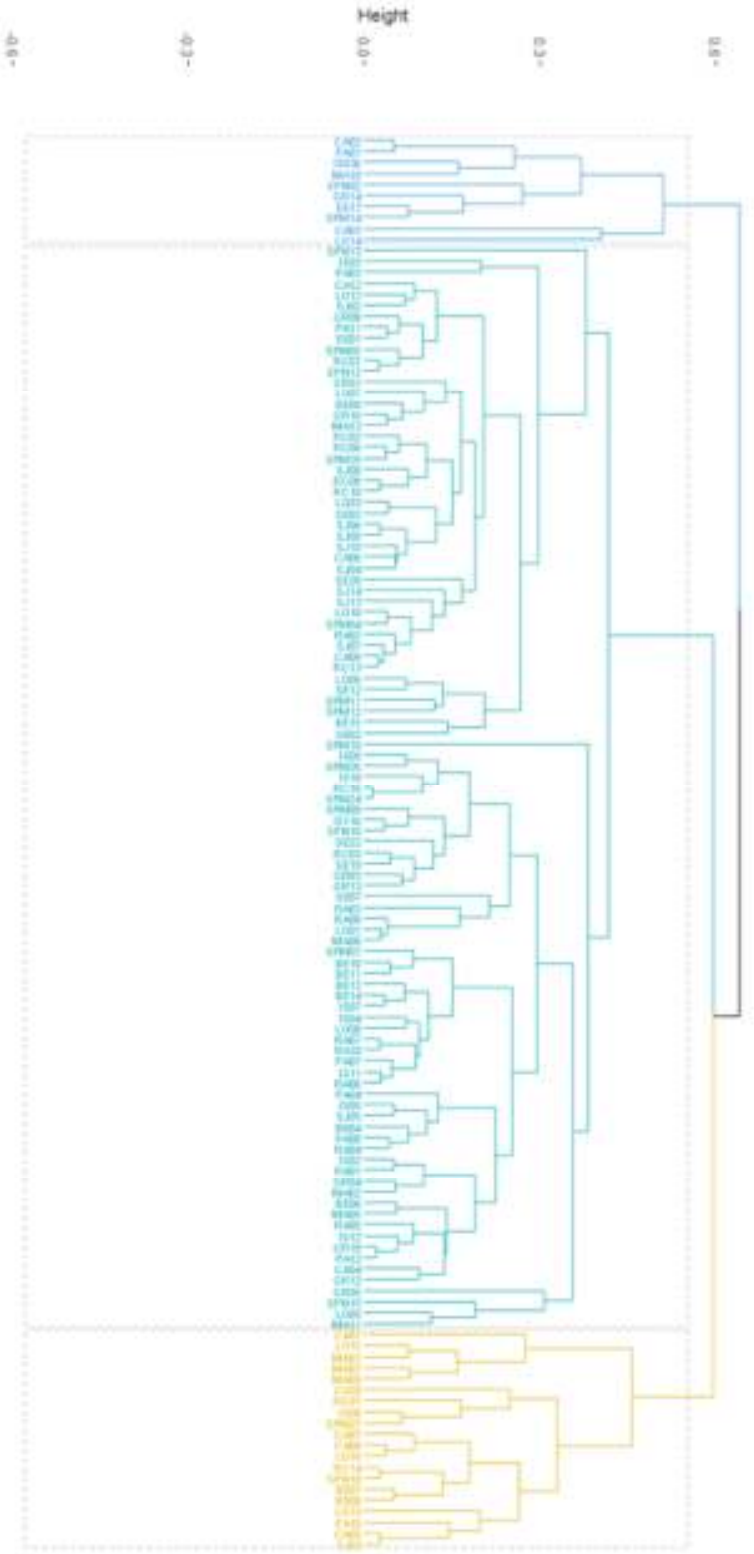

Figure 6.16 Average based dendrogram for transcription levels of all immune genes analysed in CSL pup samples.

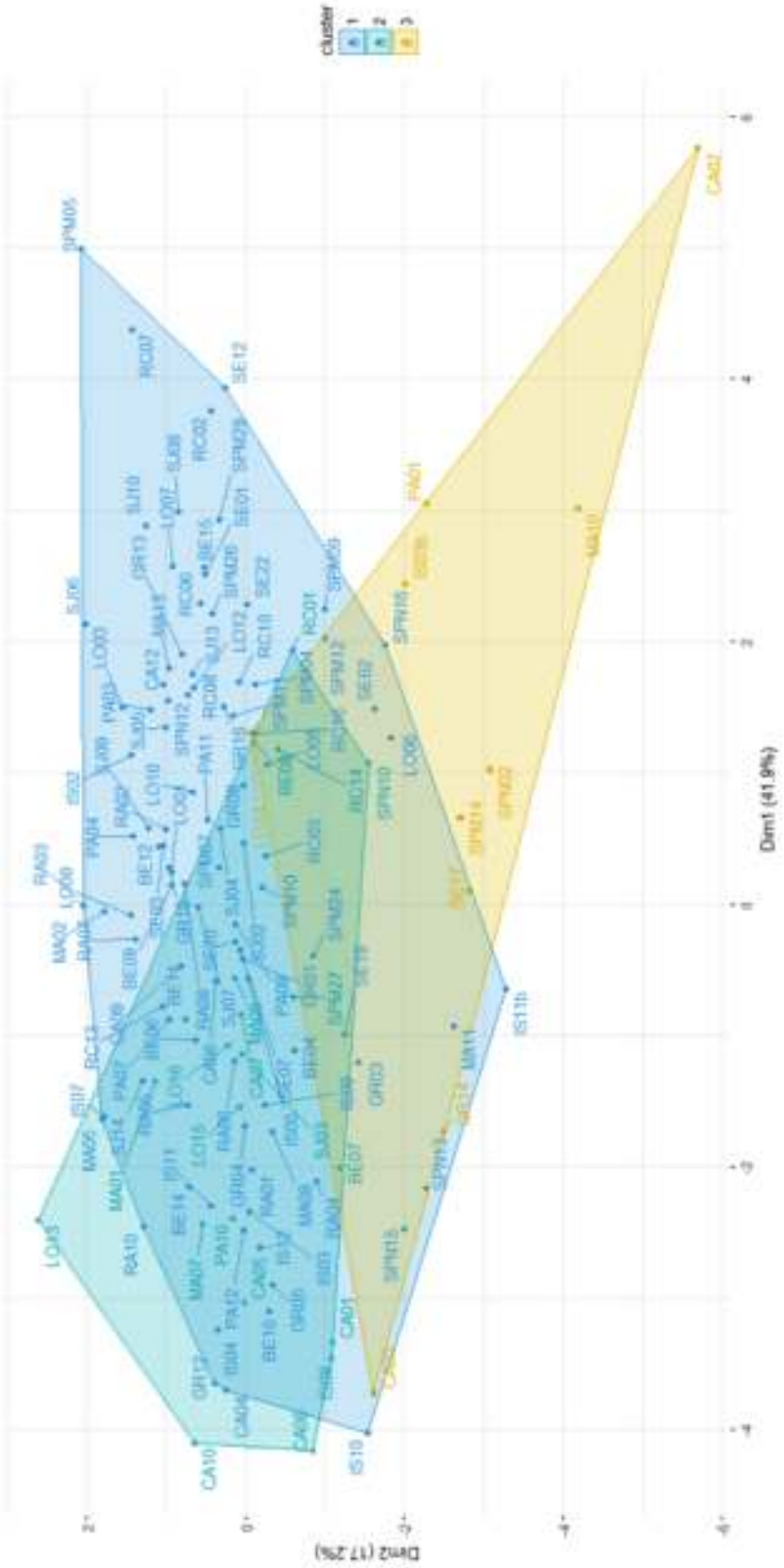

Figure 6.17 Cluster analysis of CSL pup immune gene transcription.

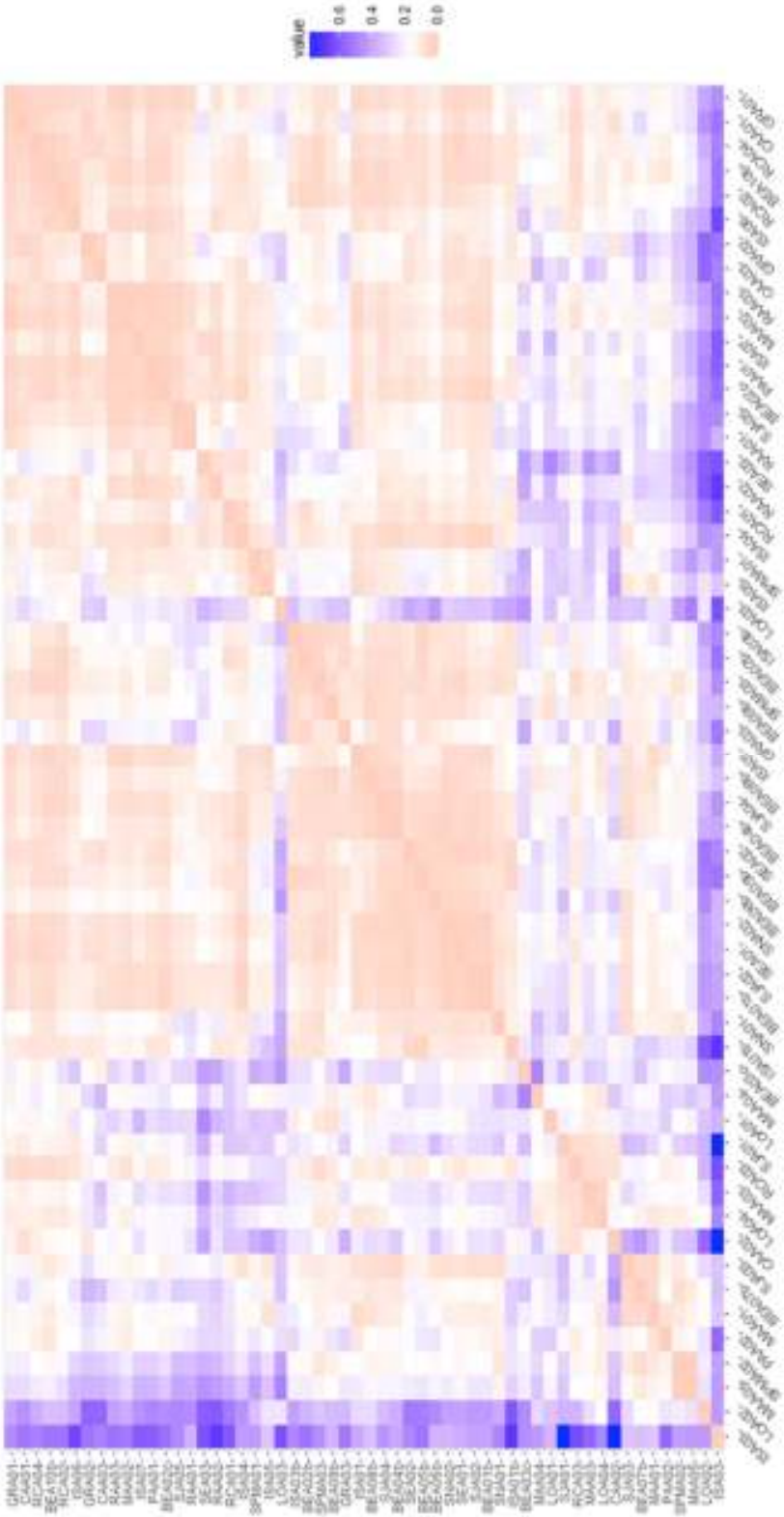

Figure 6.18 Heat map of transcription levels for all immune genes in adult CSL.

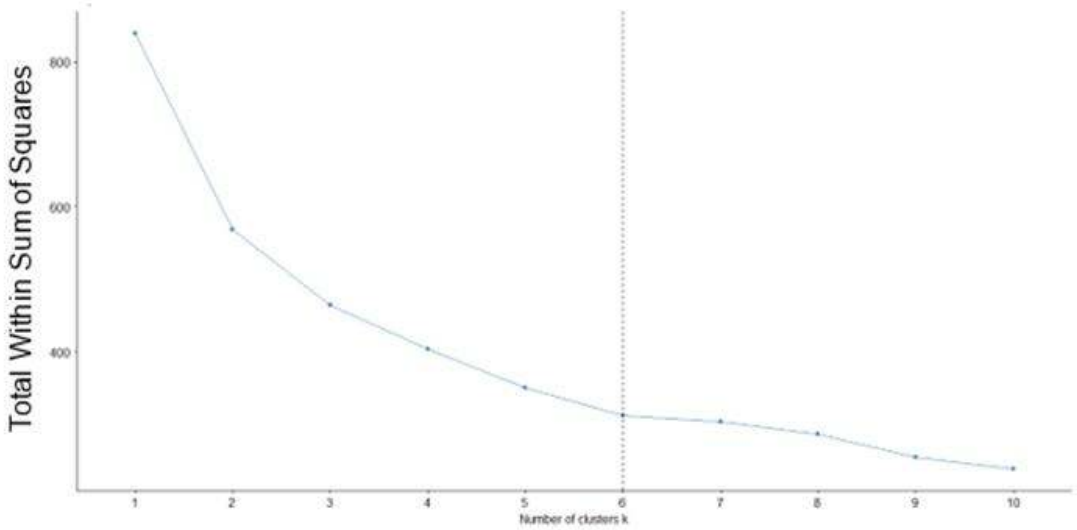

Figure 6.19. Rarefaction curve considering transcription levels of all immune genes analysed for adult CSL. The dotted line shows the optimal number of clusters.

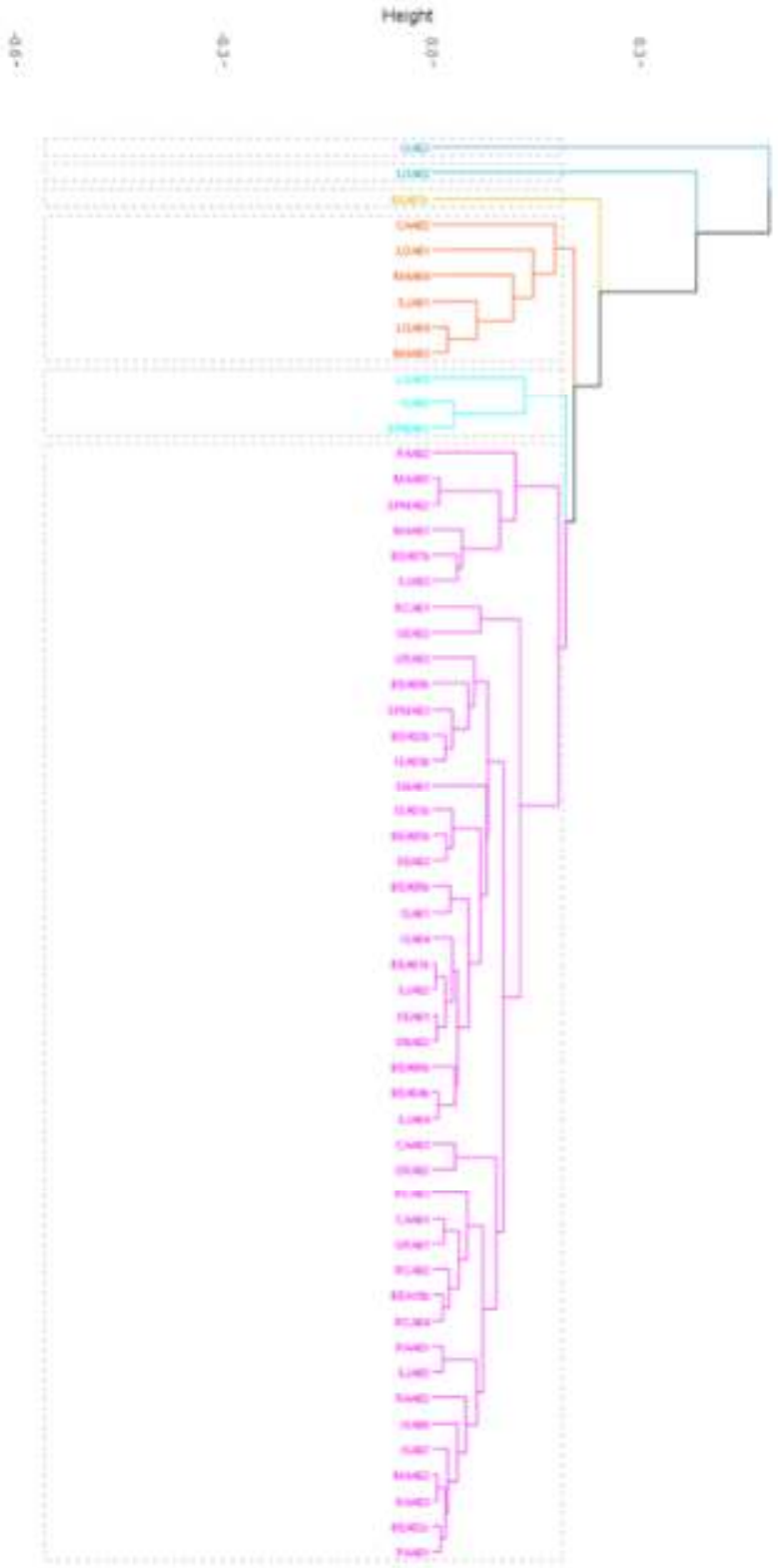

Figure 6.20. Average based dendrogram for transcription levels of all immune genes analysed in adult CSL.

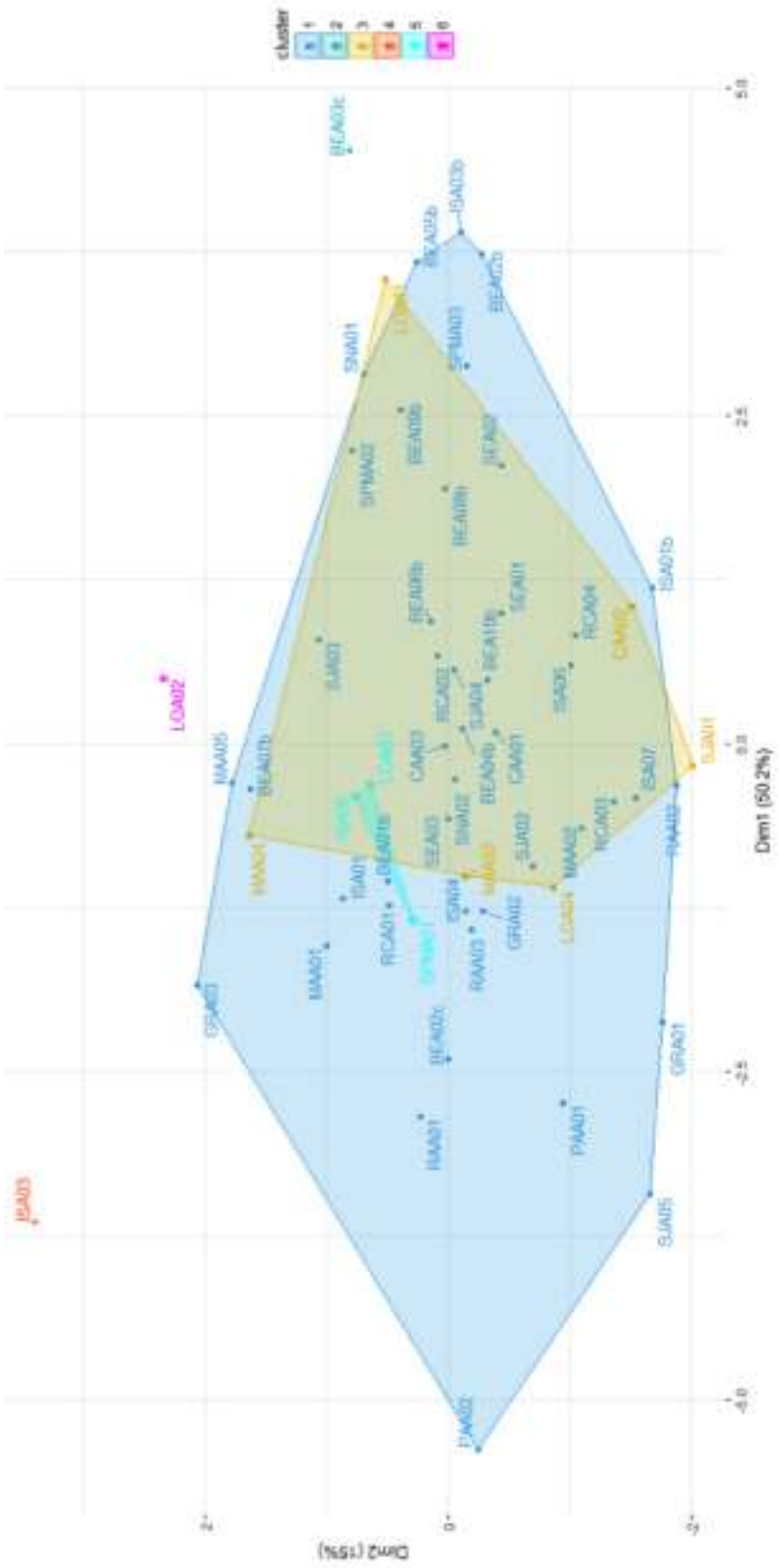

Figure 6.21 Cluster analysis for transcription levels of all immune genes analysed in adult CSL.

As mentioned earlier, when considering all of the genes in one same analysis, there was no evidence of grouping among individuals. Thus, I fragmented the analysis to group genes by functional responses. The first analysis was done including only genes indicative of the Th1 response in pup samples. The heatmap for pup data was not built, as it could only include data recorded for two genes (Stat-1 and Tbet). However, an initial rarefaction analysis using within sum of squares indicated that there were four groups (Fig. 6.22) that were represented in a Euclidean dendrogram (Fig. 6.23). The Th1 response grouped into four clusters, separating pups that had a high response and those which had a low response. Although there was no discernible geographical pattern, the colony Cantiles was overrepresented among the samples that exhibited a low response, while colonies San Pedro Mártir and Rocas Consag each had four animals with a high response (Fig. 6.24).

For adult CSL, a Euclidean analysis was used instead of ward.2 and average algorithm in the analysis for Th1 responses, as only two genes were included. Three groups were determined in the rarefaction analysis with the within sum of squares method and were represented in a dendrogram (Fig. 6.25 and 6.26). The clustering revealed a group of southern and Pacific colonies showing higher Tbet and Stat-1 responses. Only five animals from Benitos, Islotes and San Pedro Mártir were included in the low expression group, which was mainly conformed of individuals belonging to the northern and central colonies. However, most of the samples were clustered in the central group, which seemed to have an average response for both genes (Fig. 6.27).

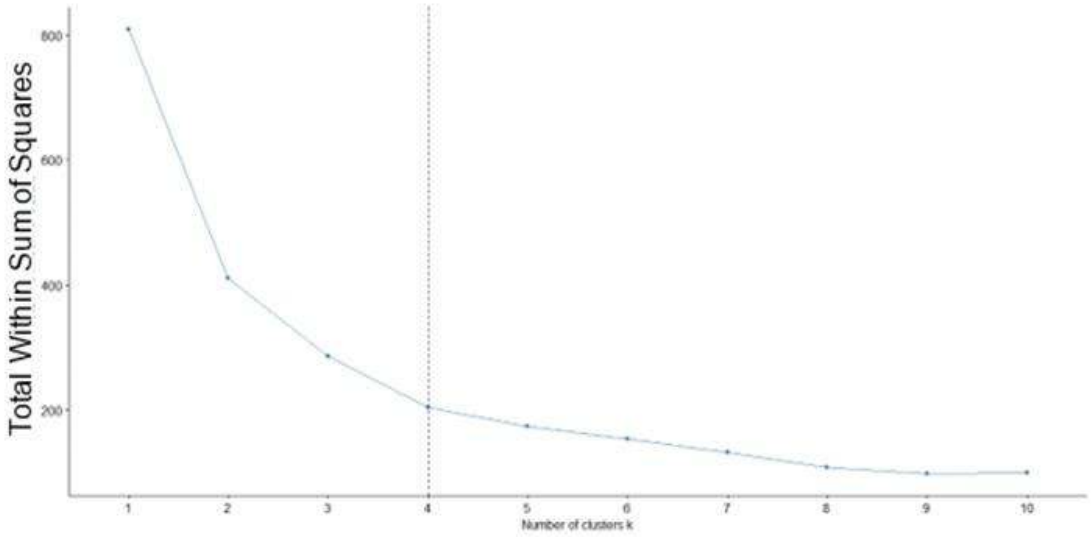

Figure 6.22 Rarefaction curve considering Th1-response genes in CSL pups. The dotted line shows the optimal number of clusters.

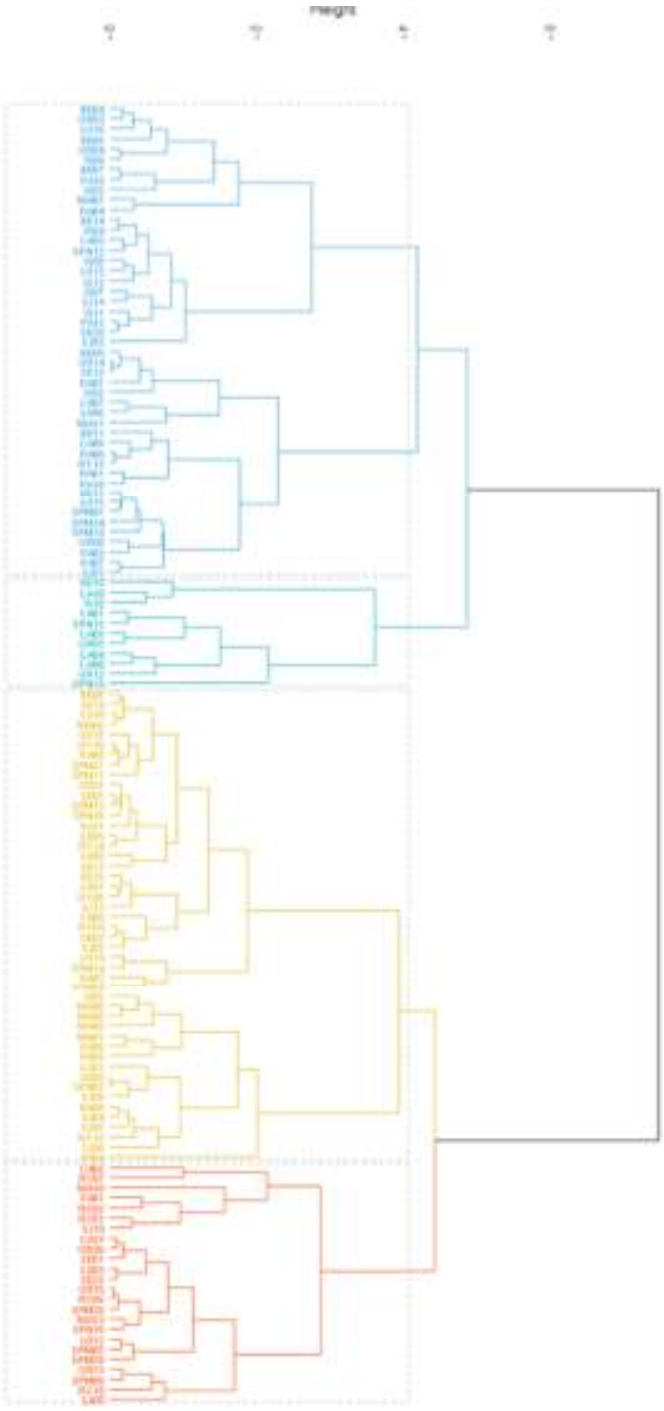

Figure 6.23 Euclidean based dendrogram for Th1-response gene transcription in CSL pups.

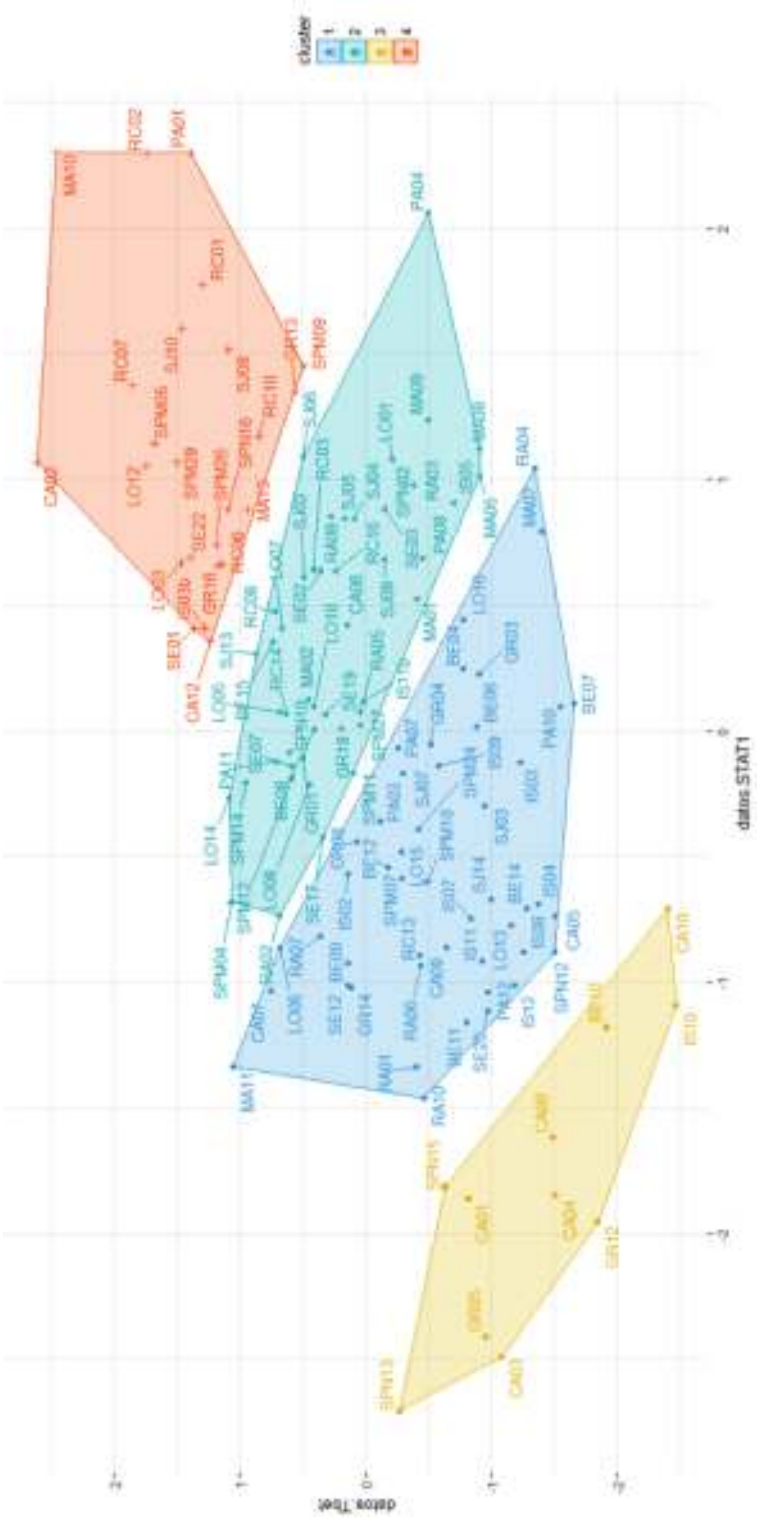

Figure 6.24 Cluster analysis of Th1-response gene transcription of CSL pups.

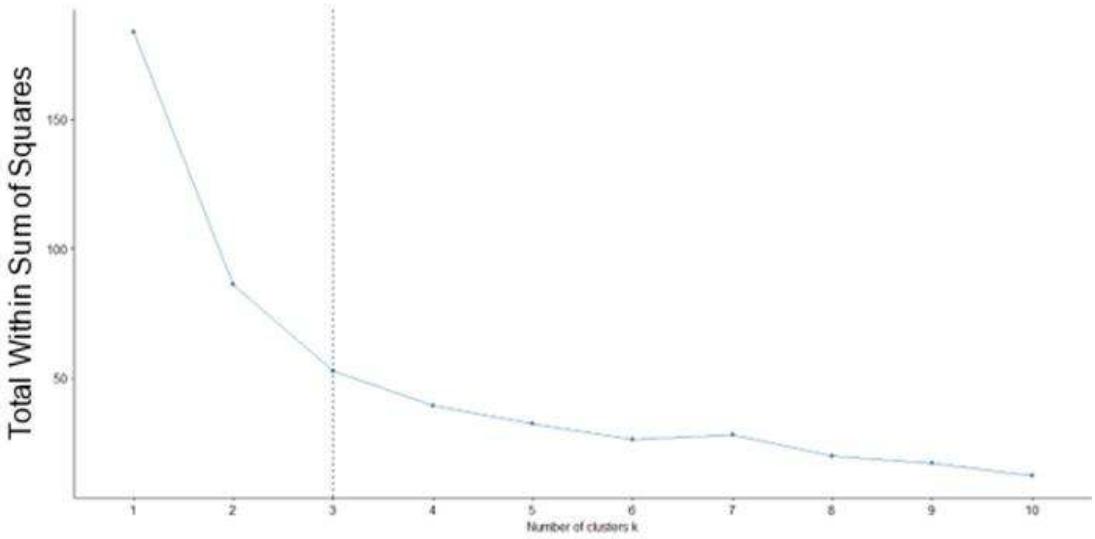

Figure 6.25 Rarefaction curve considering Th1-response gene transcription in adult CSL. The dotted line shows the optimal number of clusters.

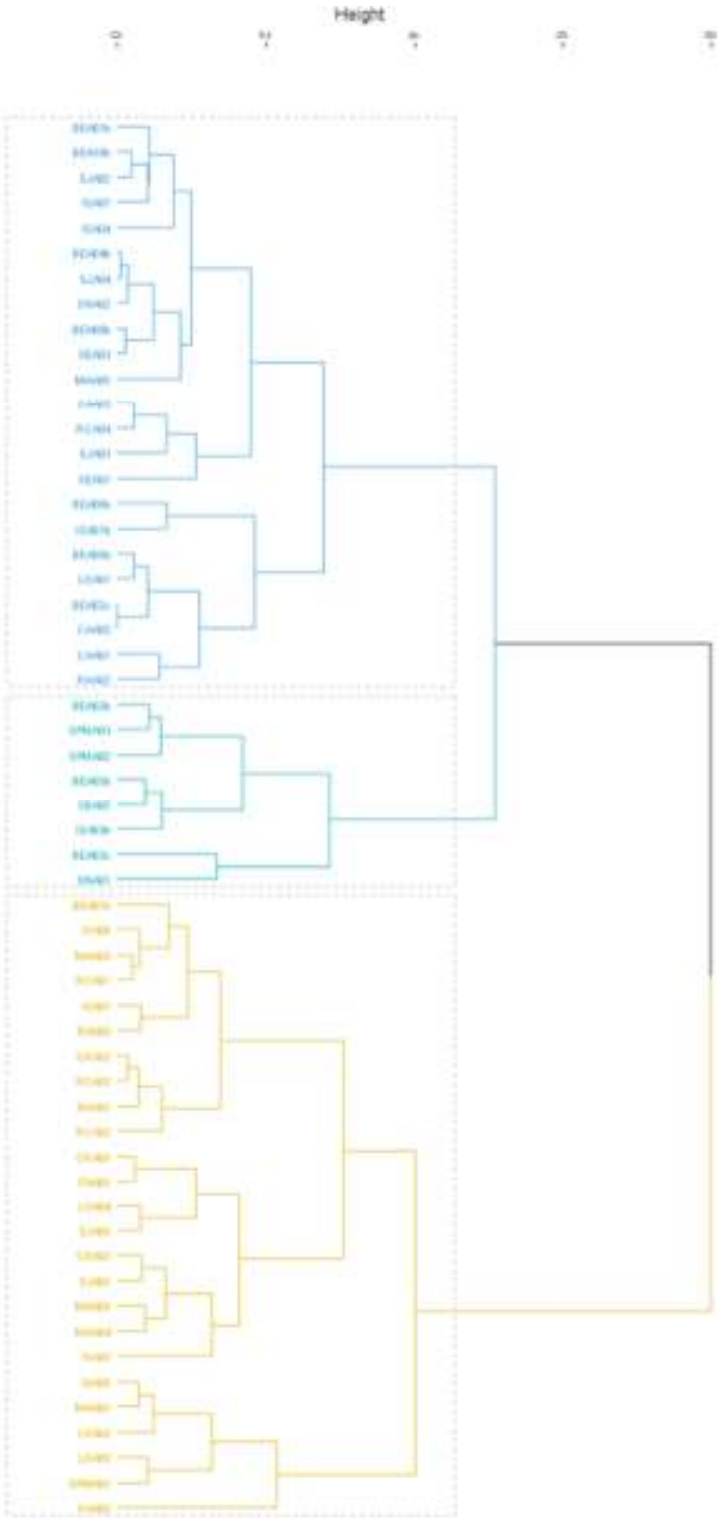

Figure 6.26 Euclidean based dendrogram for Th1-response gene transcription in adult CSL.

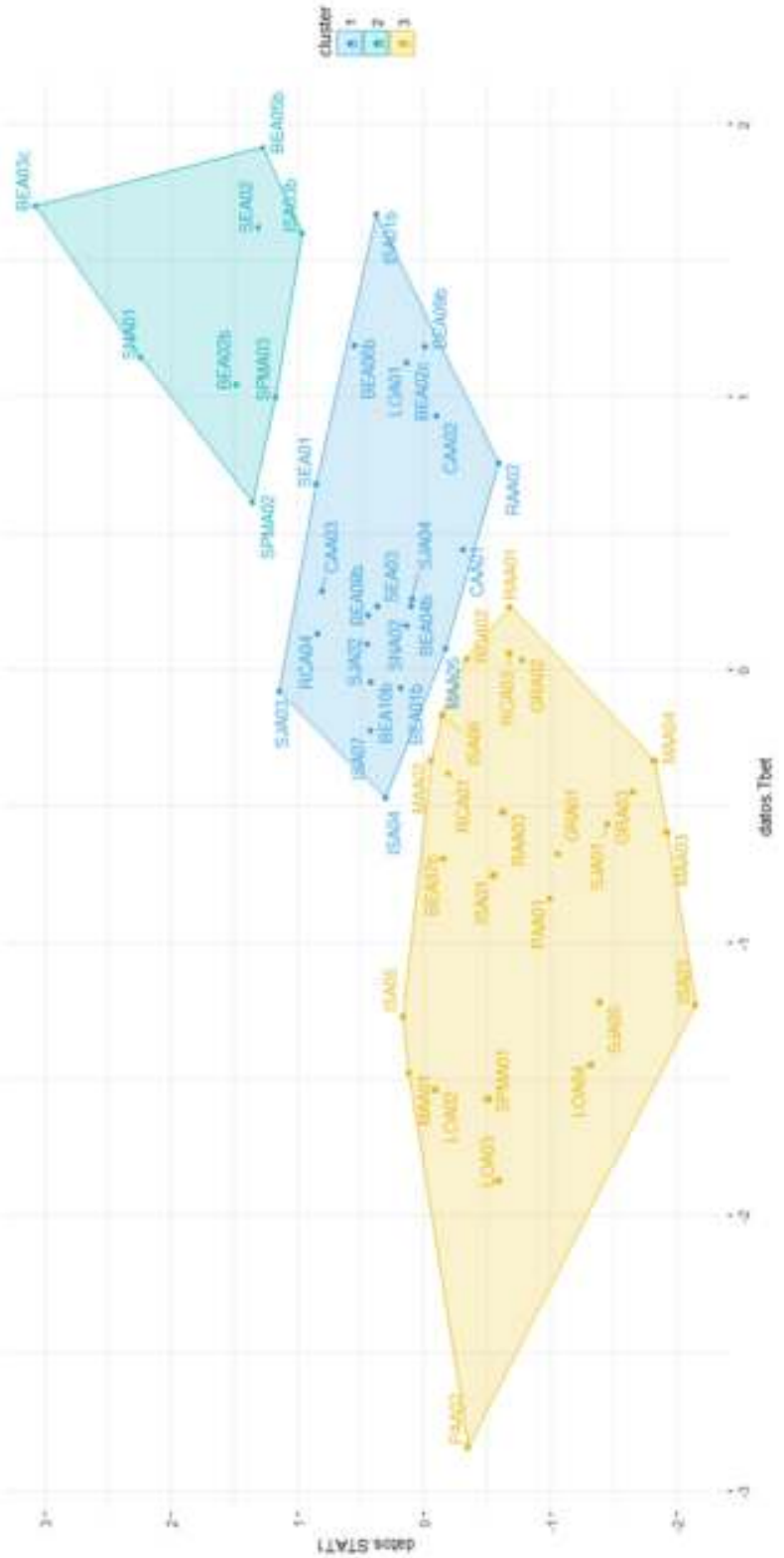

Figure 6.27 Cluster analysis of Th1-response gene transcription in adult CSL.

As occurred when analysing the Th1 response of pups, the Th2 response of pups could not be measured with ward.2 and average measurements, and the heat map was not informative. Thus, the Euclidean method was used to build the dendrogram and show the number of groups to include. Three groups were clearly defined (Fig. 6.28 and 6.29) although they did not correspond to any particular geographical grouping. Individuals from San Pedro Mártir and Rocas Consag were overrepresented in group 3, which appeared to have a greater Th2 response (Fig. 6.30). This was similar to what was observed for the Th1 response.

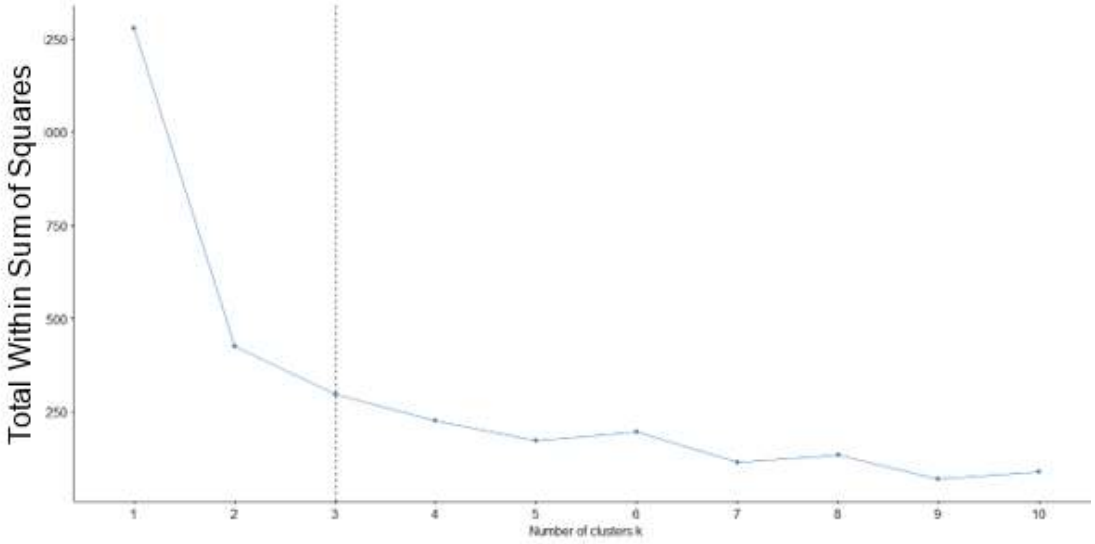

Figure 6.28 Rarefaction curve considering Th2-response genes in CSL pups. The dotted line shows the optimal number of clusters.

As there was only data for one Th2-response gene (GATA3) available for adult CSL, it was not possible to analyse clustering of gene transcription for this functional response. Thus, in this case, only the results obtained by the GLM were considered (see above).

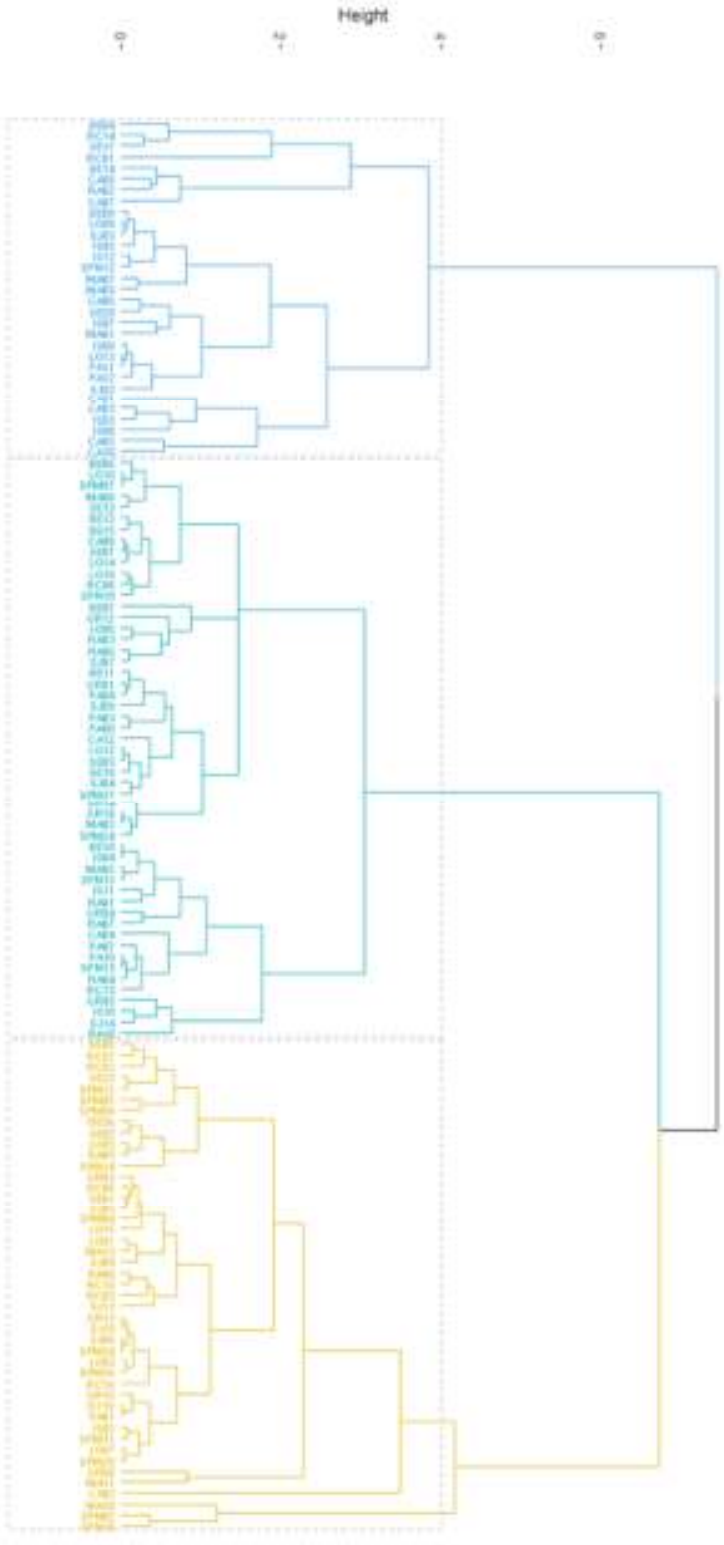

Figure 6.29. Euclidean based dendrogram for Th2-response genes in CSL pups.

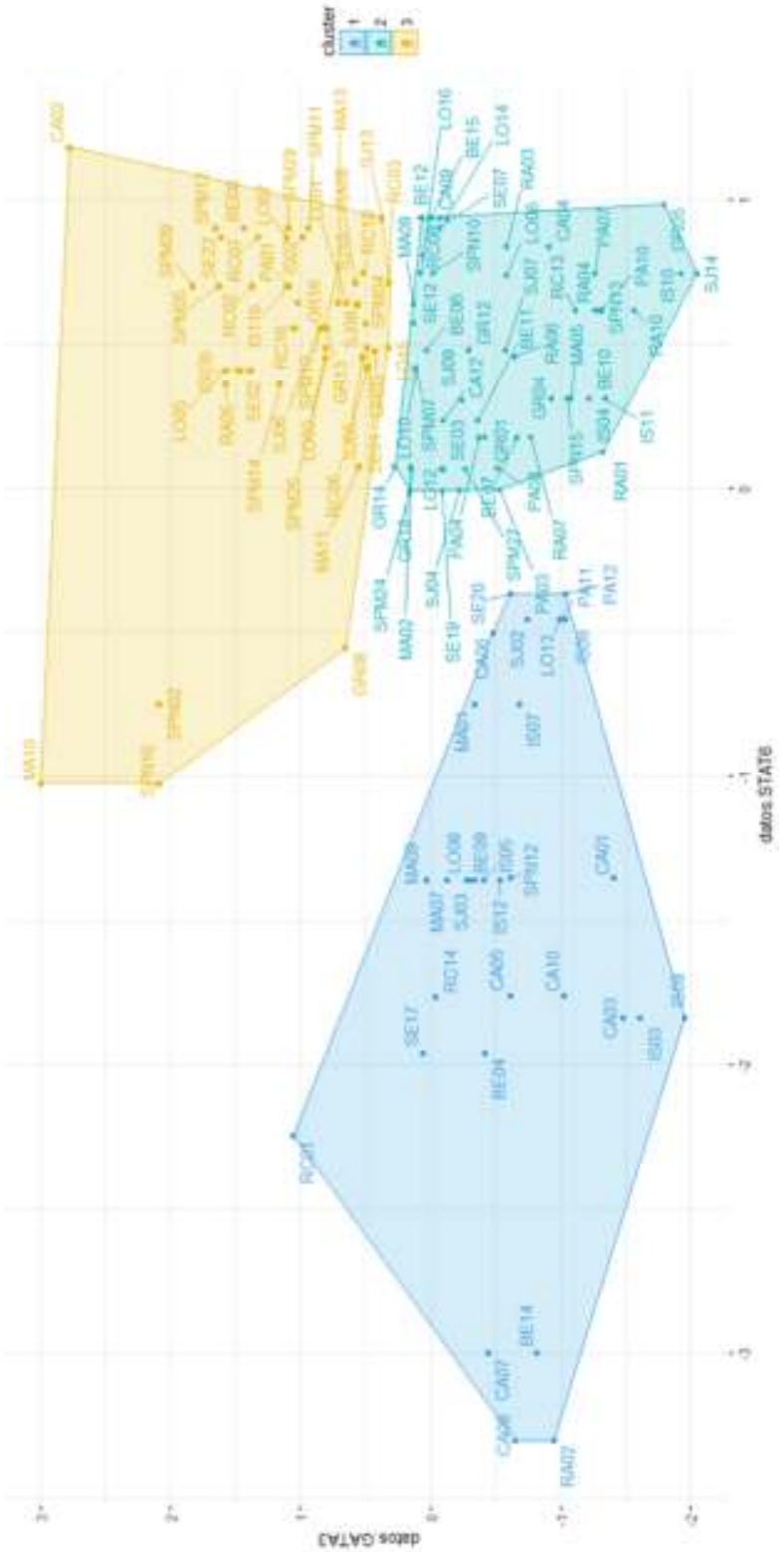

Figure 6.30 Cluster analysis of Th2-response genes from CSL pups.

Transcription levels of STAT-1 and GATA3 in both pups and adults were correlated in the preliminary analysis, and they were included in CD4 response that included Th1 and Th2 responses, to determine if this lymphocyte population as a whole is responsible of clustering. The ward.2 algorithm had low explanatory power for pup data according to the cophenetic correlation (0.48) but the average method was very powerful (0.84) and was thus used to build a four-group dendrogram (Fig. 6.31 and 6.32). The cluster analysis, built using the two main components, explained over 90% of the variability, but the groups formed did not have a geographical correspondence. The most distant group consisted in a mixture of pups from the northern, central and southern colonies and was defined by the second PC (Fig. 6.33).

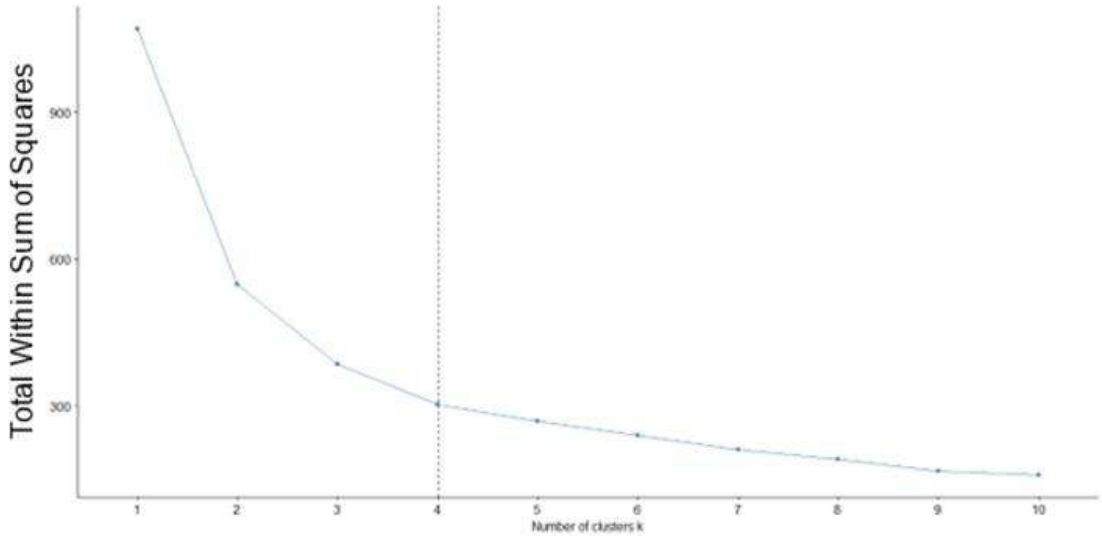

Figure 6.31. Rarefaction curve considering transcription levels of genes involved in CD4 T cell function of CSL pups. The dotted line shows the optimal number of curves.

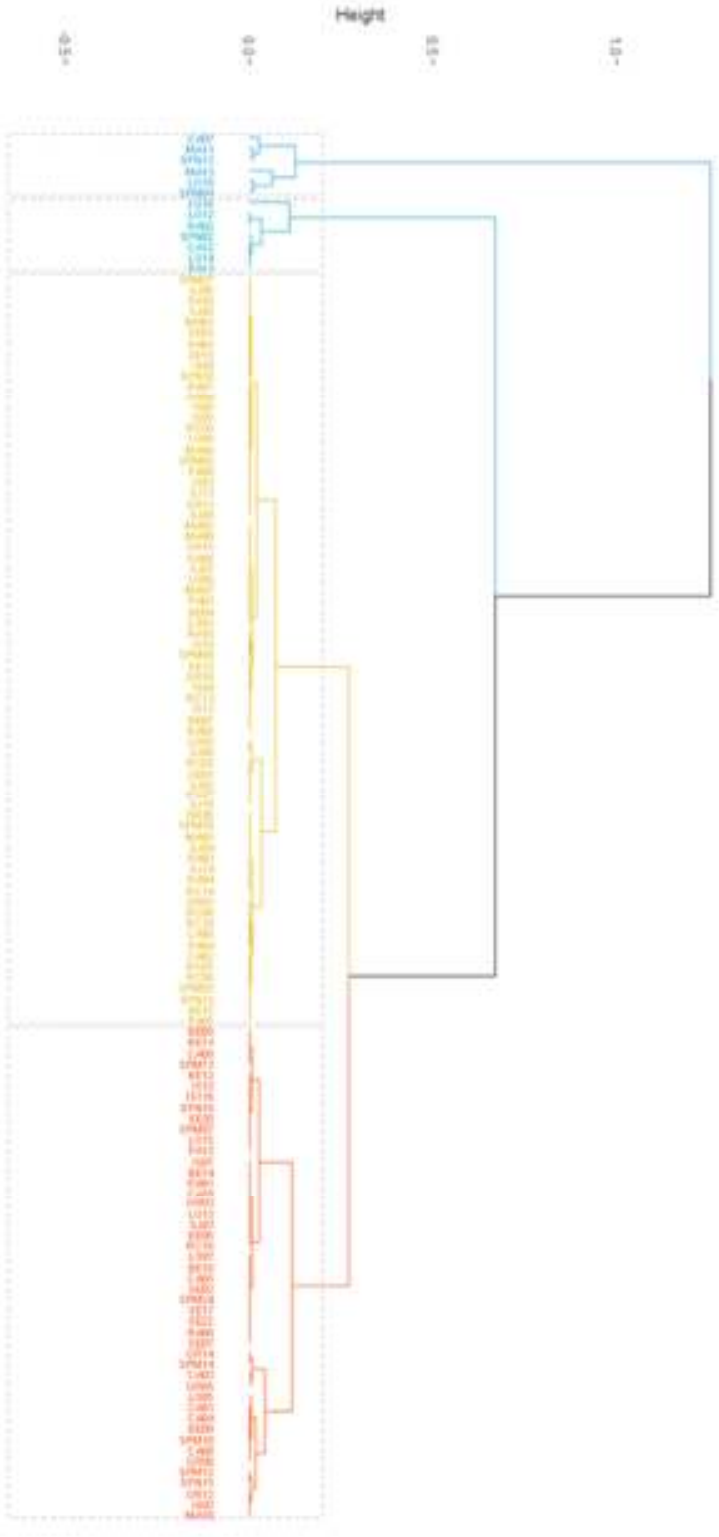

Figure 6.32. Average based dendrogram for transcription levels of genes involved in CD4 T cell function of CSL pups.

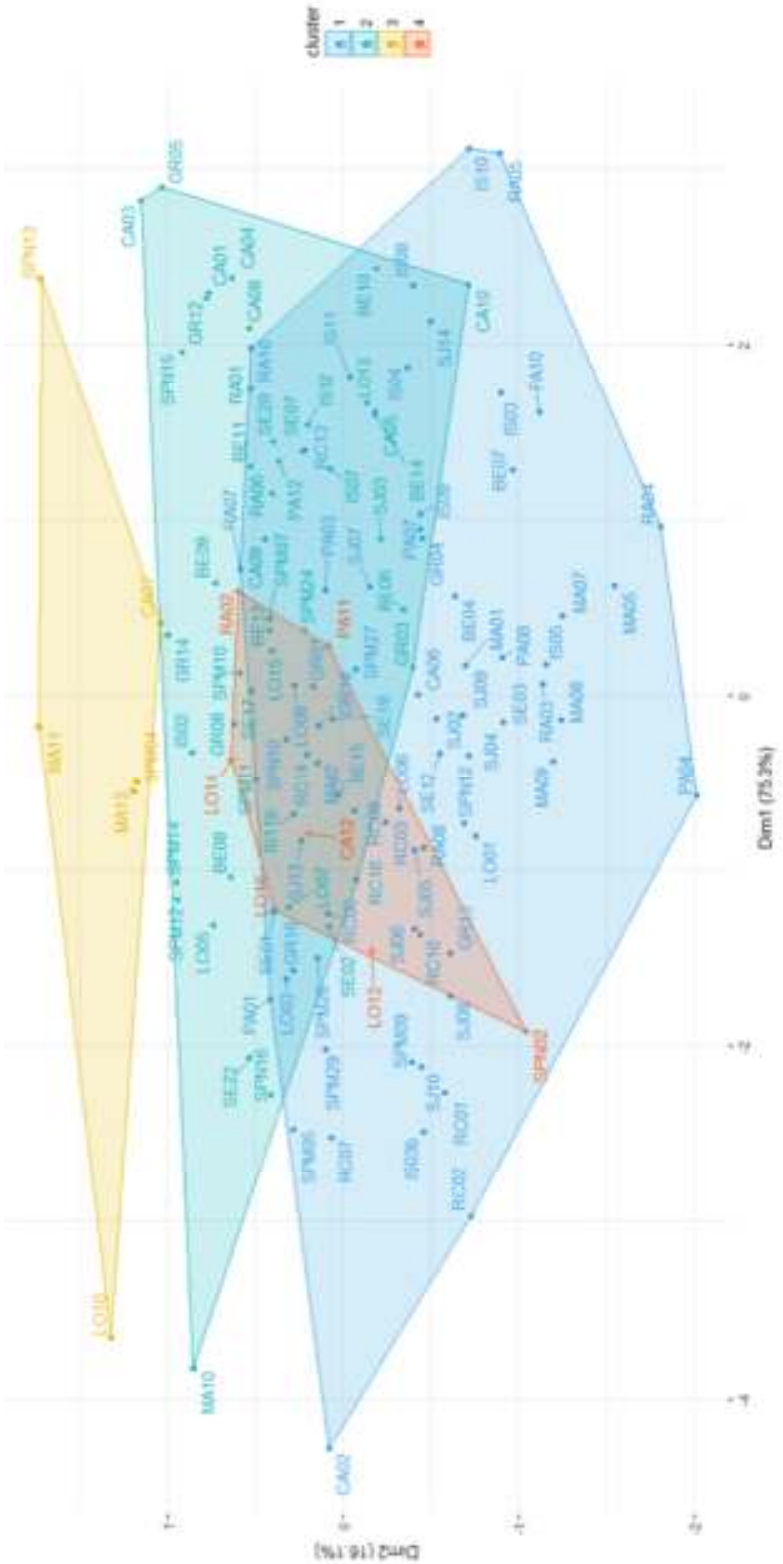

Figure 6.33 Cluster analysis of transcription levels of genes involved in CD4 T cell function of CSL pups.

The transcripts STAT1 and GATA3 considered together were also used for the analysis of data obtained for adult CSL. The heat map seemed to define three to four groups (Fig. 6.34), and three groups were also obtained in the rarefaction analysis (Fig. 6.35). The values of correlation for the analysis using ward.2 (0.7) and average (0.75) were close to the minimum considered representative. As it has a higher correlation level, the second one was selected to graph the dendrogram.

CD8 T-lymphocyte function, defined by its transcription factor Eomes and the enzymes perforin and granzyme, was subjected to the same analysis. The heat map of CD8 activity of pups showed an intense correlation of a few samples. The other samples showed undetectable associations (Fig. 6.36). The ward.2 dendrogram was less significant (0.63) than the average (0.79), according to the cophenetic correlation. Thus, the number of groups determined by rarefaction (Fig. 6.37) was represented in the average dendrogram (Fig. 6.38). The cluster analysis showed, with 86% of explanatory power, an overrepresentation of samples from Rocas Consag in one of the extreme groups. Three animals in a group of eight belonged to this island (Fig. 6.39). The other two groups were massive and showed no clear representation of islands.

In adults, the heat map of CD8 transcription levels showed similarity in the expression patterns of two samples (ISA03 and SPM02) but was inconclusive for the other individuals (Fig. 6.40). The rarefaction analysis showed bends at 3 and 5 groups (Fig 6.41). Thus, I decided to graph both options. The average based dendrogram (0.80) was more informative than the dendrogram using ward.2 algorithm (0.60) and the dendrograms for the two grouping were represented. The dendrogram with three groups was represented in Fig. 6.42 and the clusters in which (ISA03 and SPM02) are isolated are shown in Fig. 6.43. This is similar to what was fiund for the ‘five group’

dendrogram (Fig.6.44) and its corresponding cluster (Fig. 6.45 and 6.46). In both cases, ISA03 and SPM02 grouped separately to the other samples. These samples showed an overlap.

When analysing NK function, the heat map for pups showed several groups but when genes were observed to look for a clear pattern, I found none (Fig 6.47). Rarefaction analysis reported four groups determined (Fig 6.48). The dendrogram using average algorhitm showed better cophenetic correlation (0.74) compared to ward.2 (0.57), thus, it was selected to represent the clusters (Fig. 6.49). However, this value was still below ideal for an informative dendrogram (0.75). Although most of the samples overlap, and due to that, the clusters cannot explain differences, the small groups showed some characteristics. The cluster with only eight samples included three animals from Islotes. In addition, in the two samples group, there was a CSL from San Pedro Mártir and another from Islotes (Fig 6.50).

For adult CSL, NK gene transcription revealed that two individuals, ISA03 and LOA01, were highly correlated in their pattern of activity (Fig. 6.51). The rarefaction analysis showed five groups (Fig. 6.52). To represent these groups, a dendrogram using average algorithm was selected as it showed higher cophenetic correlation (0.71) than the one generated using ward.2 (0.51), (Fig. 6.53). However, 0.71 is below desired 0.75 cophenetic correlation. Despite this lack of reliability, cluster showed only slight overlap although there were no evident geographical patterns (Fig. 6.54). Two animals, ISA03 and LOA01, had a very distinctive behavior compared to the other clusters formed. ISA03 behavior concurs with CD8 analysis.

As there was only one factor related to Treg (FoxP3), I did not analyse pups or adults with these tools.

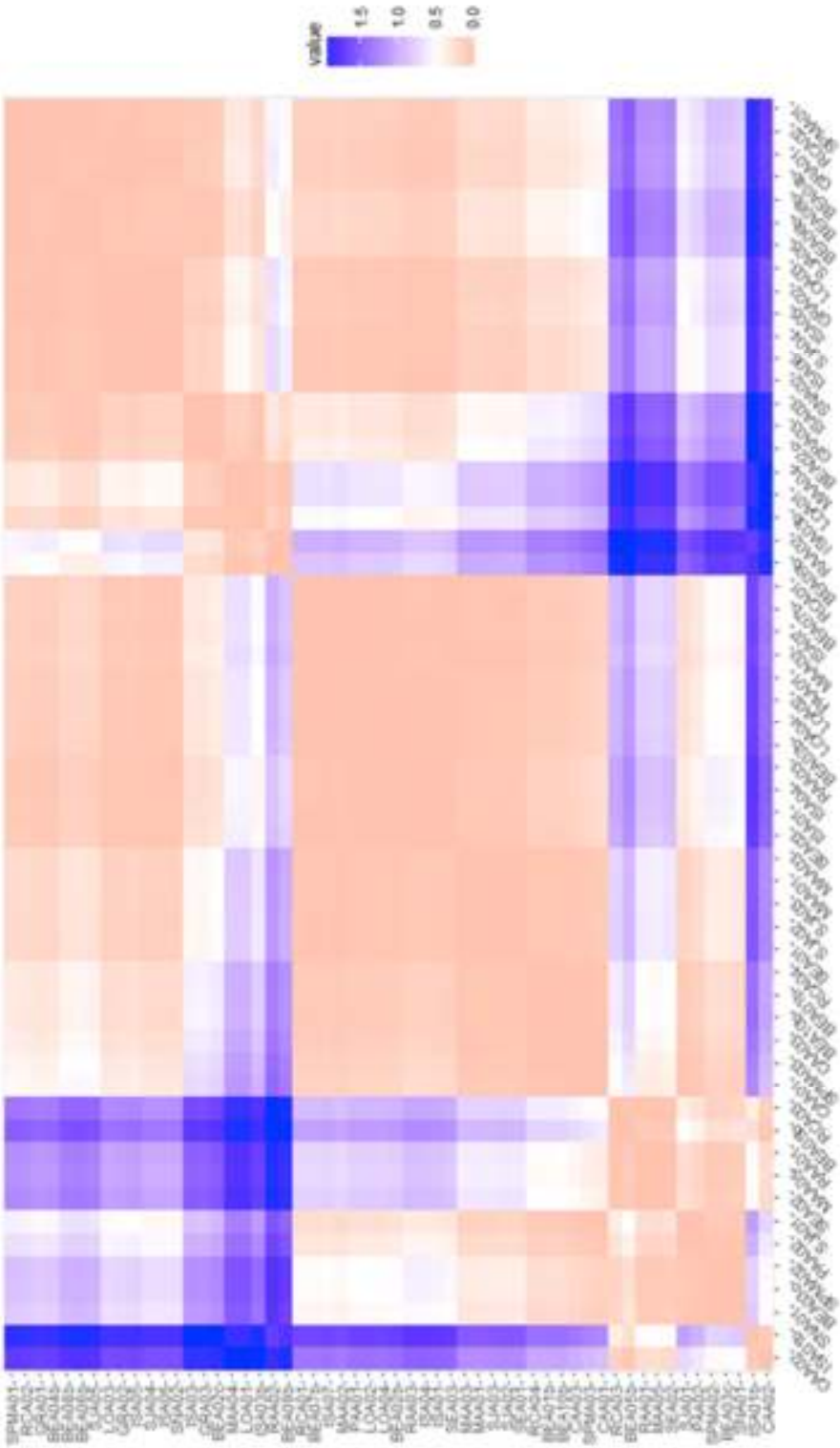

Figure 6.34 Heat map of transcription levels of STAT-1 and GATA-3 in adult CSL.

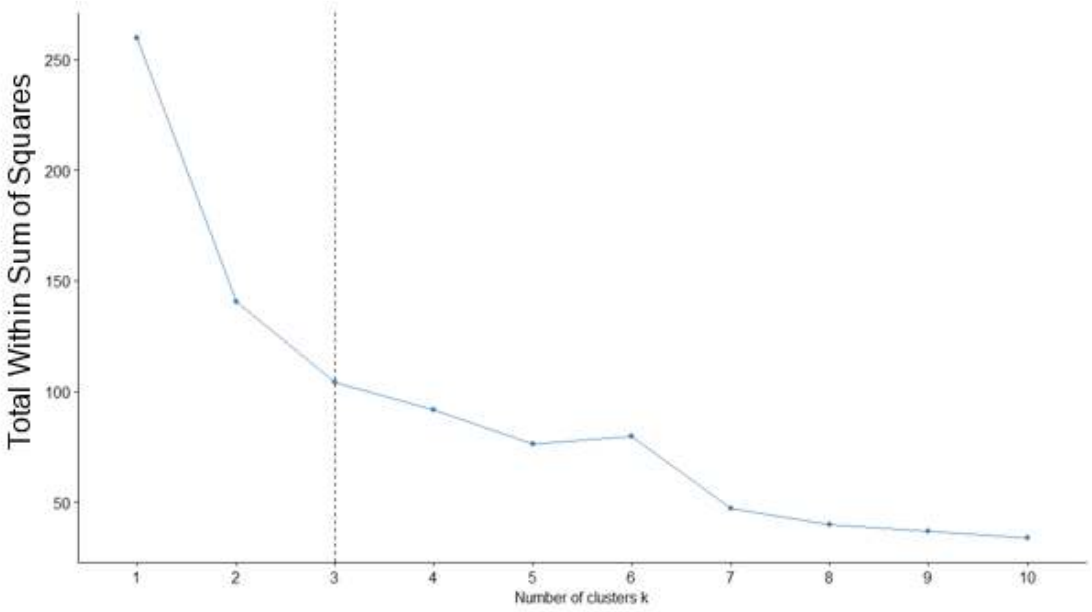

Figure 6.35. Rarefaction curve considering transcription levels of genes involved in CD4 T cell function in adult CSL. The dotted line shows the optimal number of clusters.

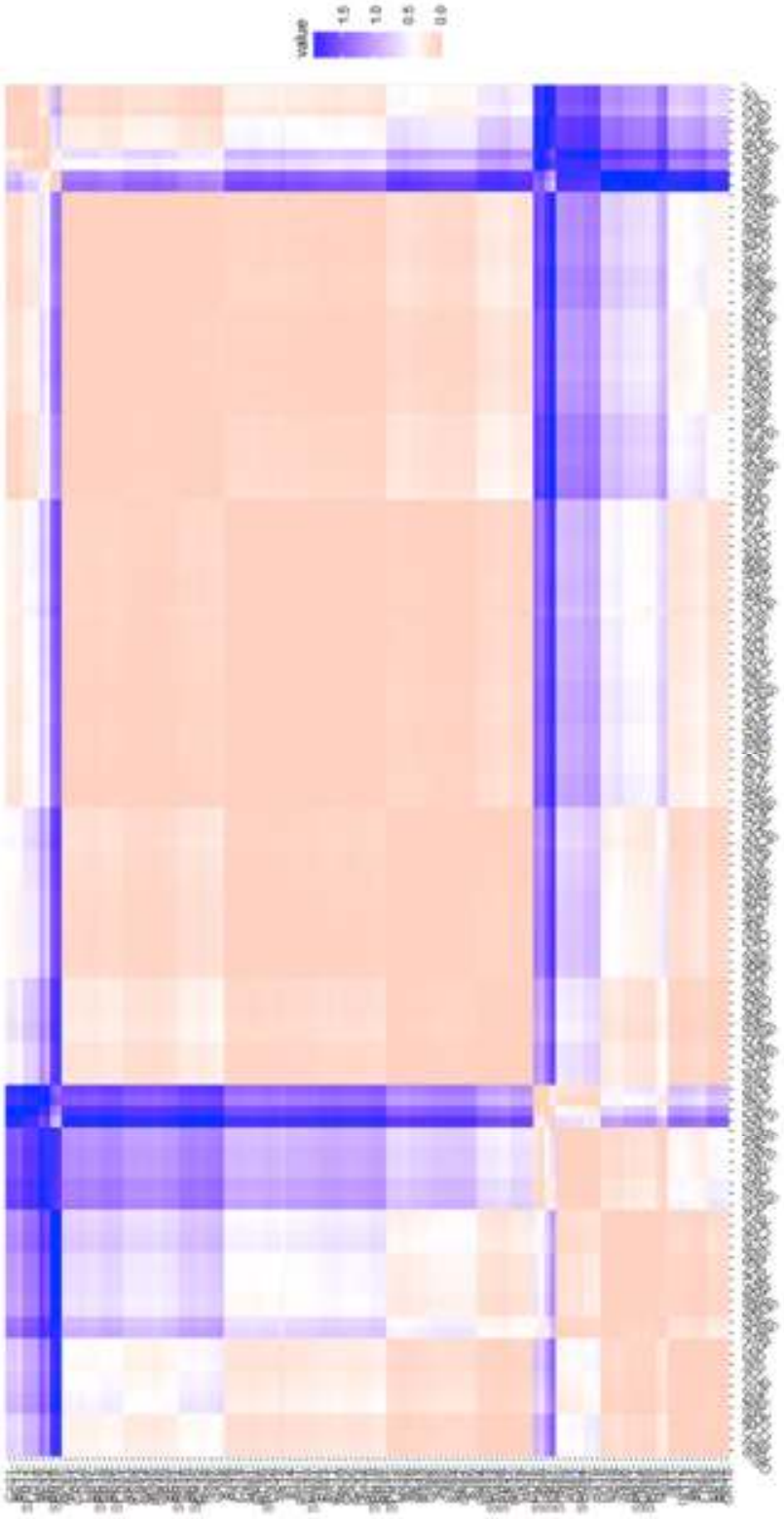

Figure 6.36 Heat map for transcription levels of genes involved in CD8 T cell function in CSL pups.

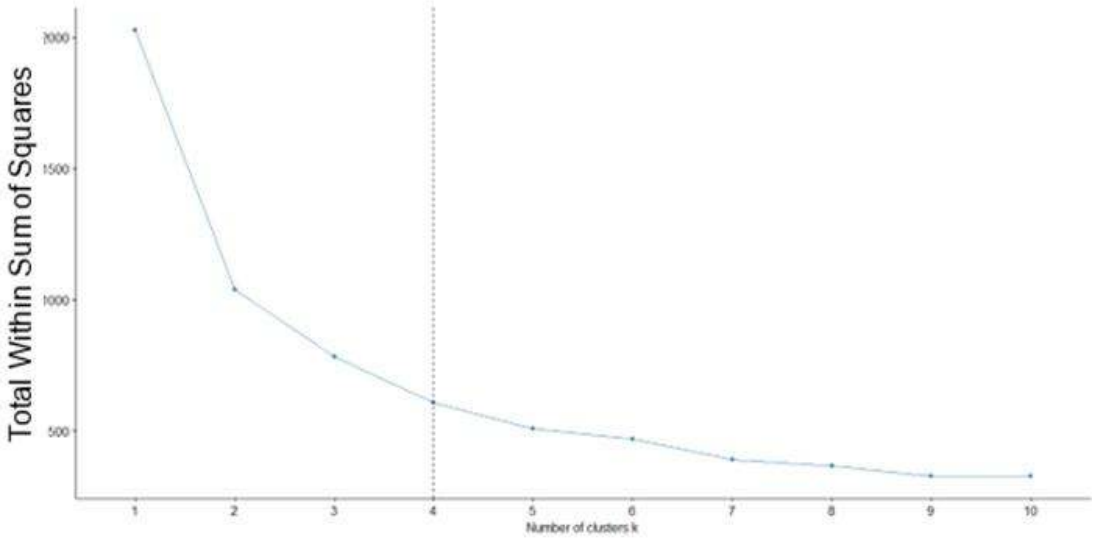

Figure 6.37 Rarefaction curve of transcription levels of genes involved in CD8 T-cell function in CSL pups. The dotted line represents the optimal bymber of clusters.

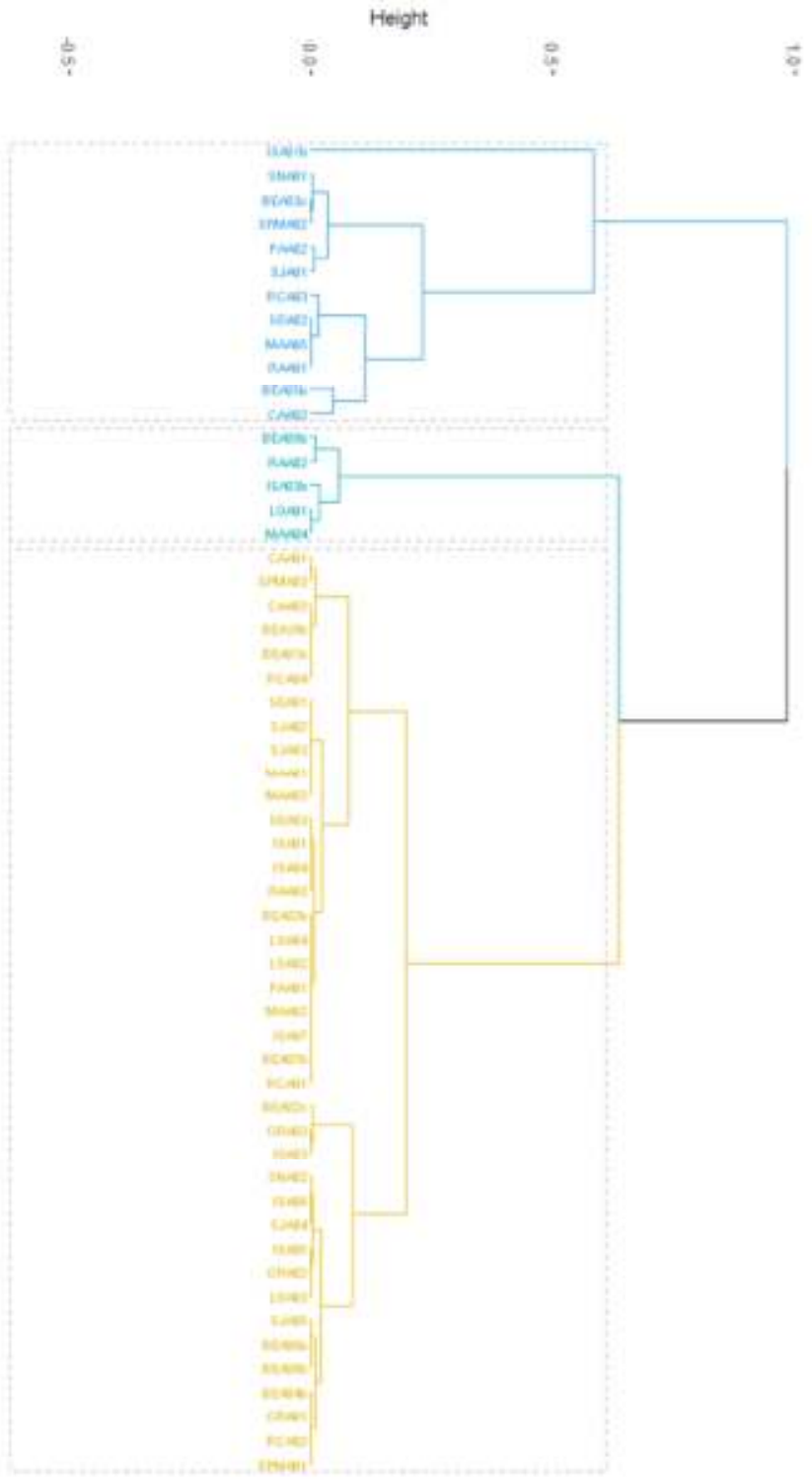

Figure 6.38. Euclidean based dendrogram of transcription levels of genes involved in CD8 T cell function of pups.

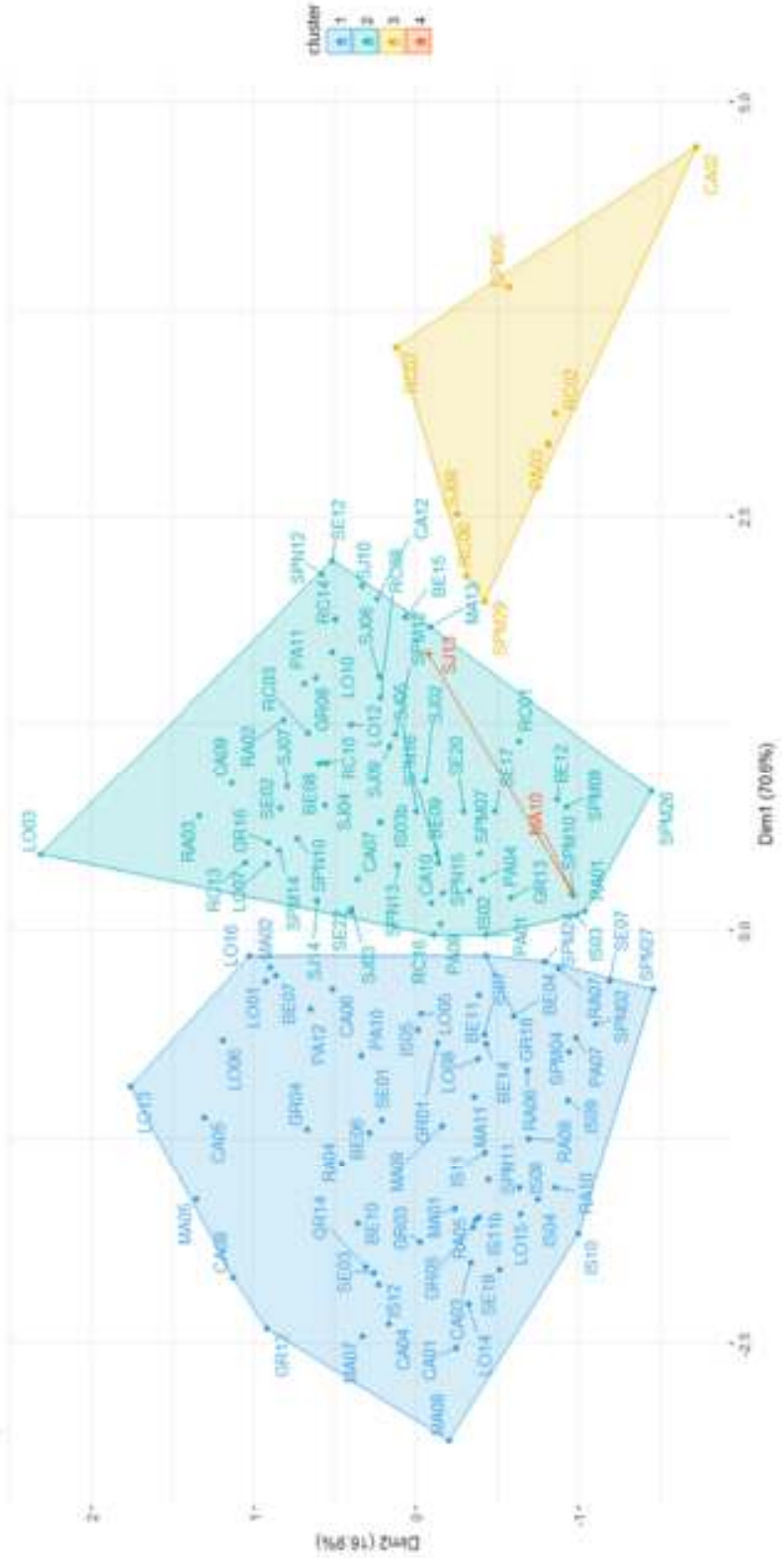

Figure 6.39. Cluster analysis of transcription levels of genes involved in CD8 T cell function in CSL pups.

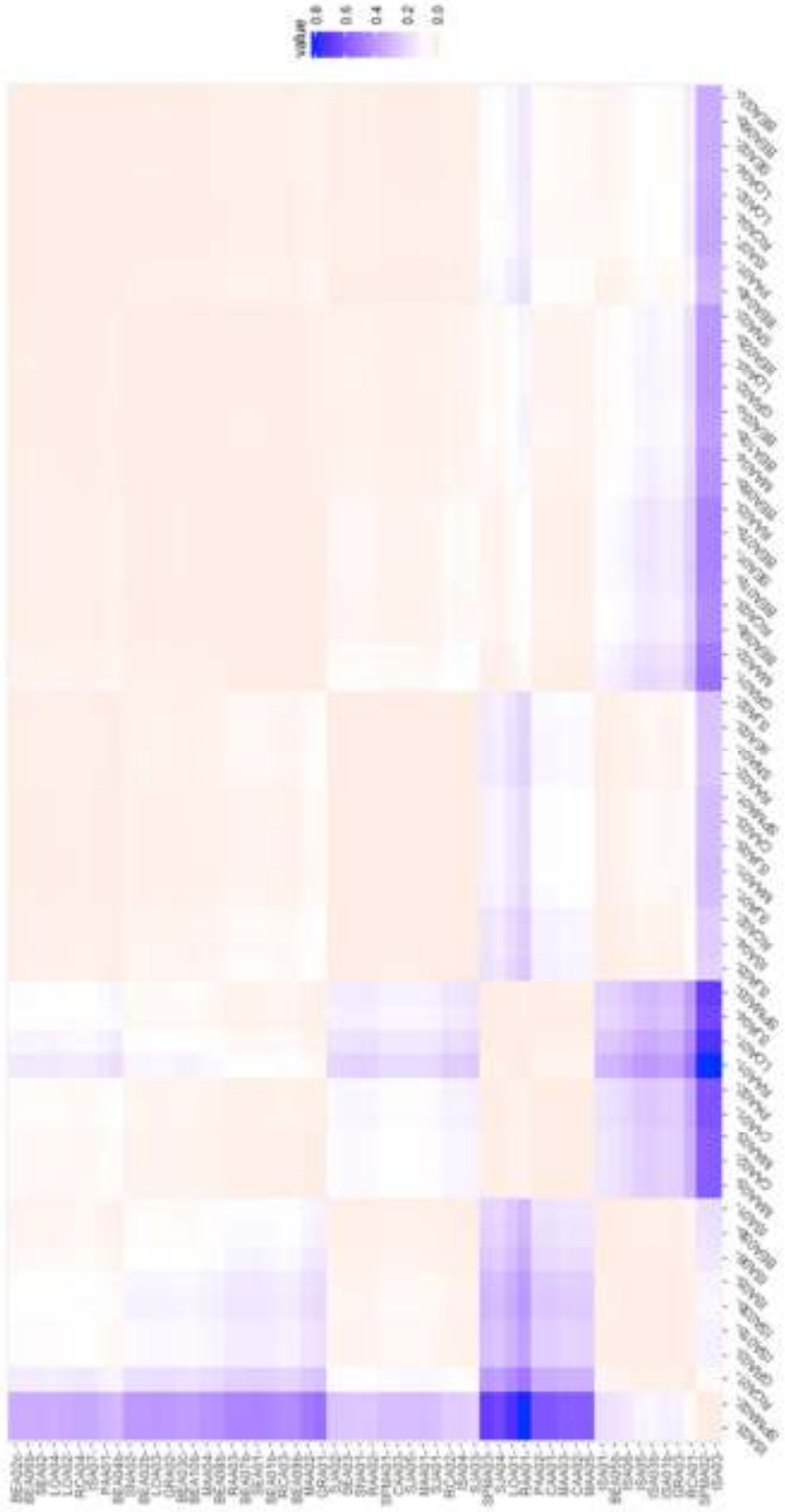

Figure 6.40 Heat map of transcription levels of genes involved in CD8 T cell function in adult CSL.

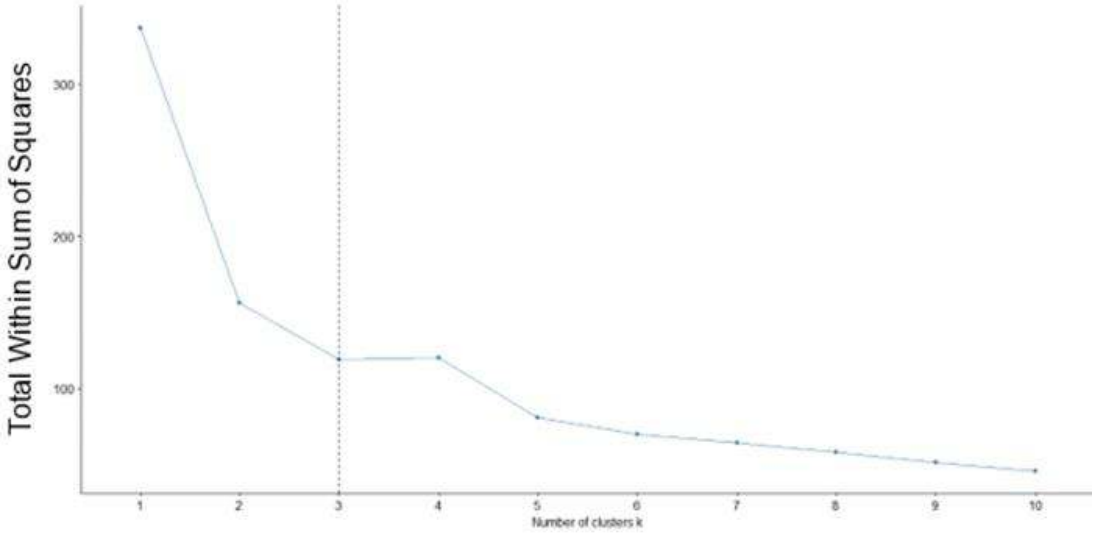

Figure 6.41 Rarefaction curve considering CD8 genes from adults. The dotted line shows the optimal number of clusters

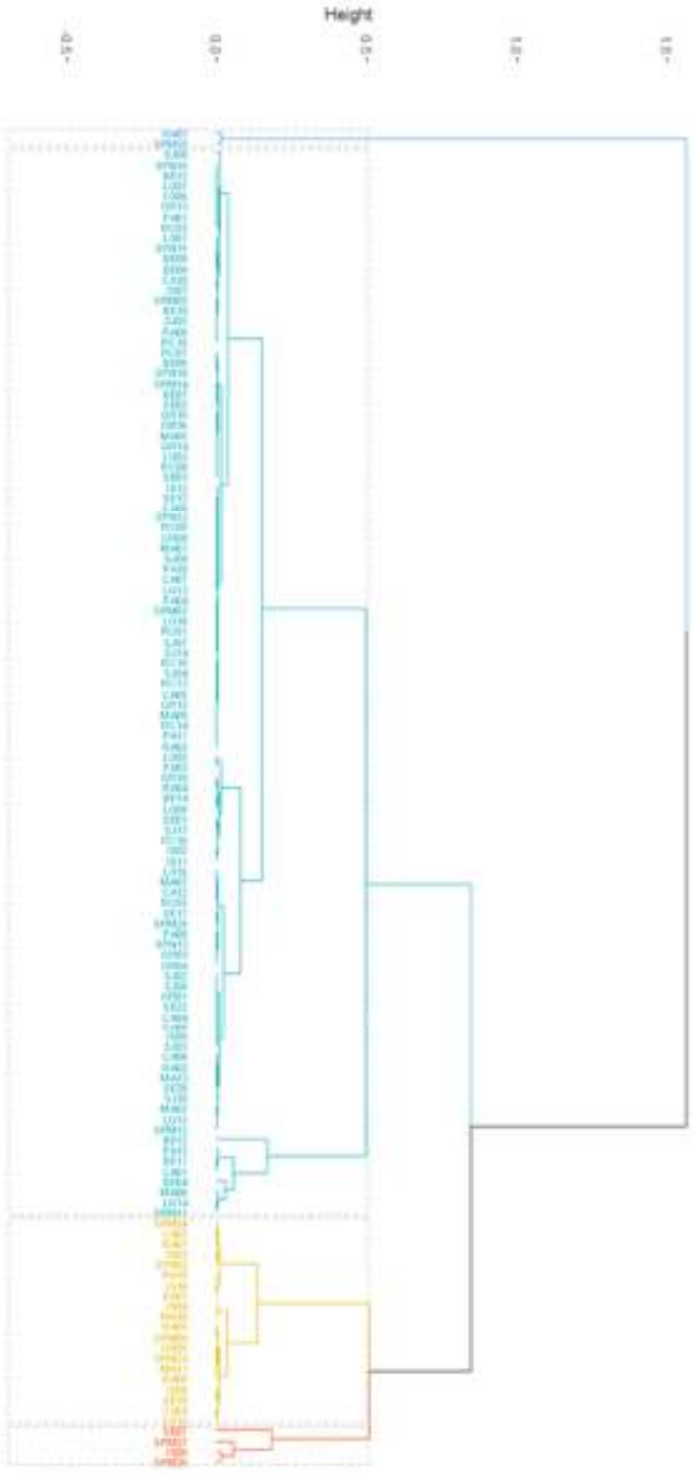

Figure 6.42. Average based dendrogram for transcription levels of genes involved in CD8 T cell function of adult CSL.

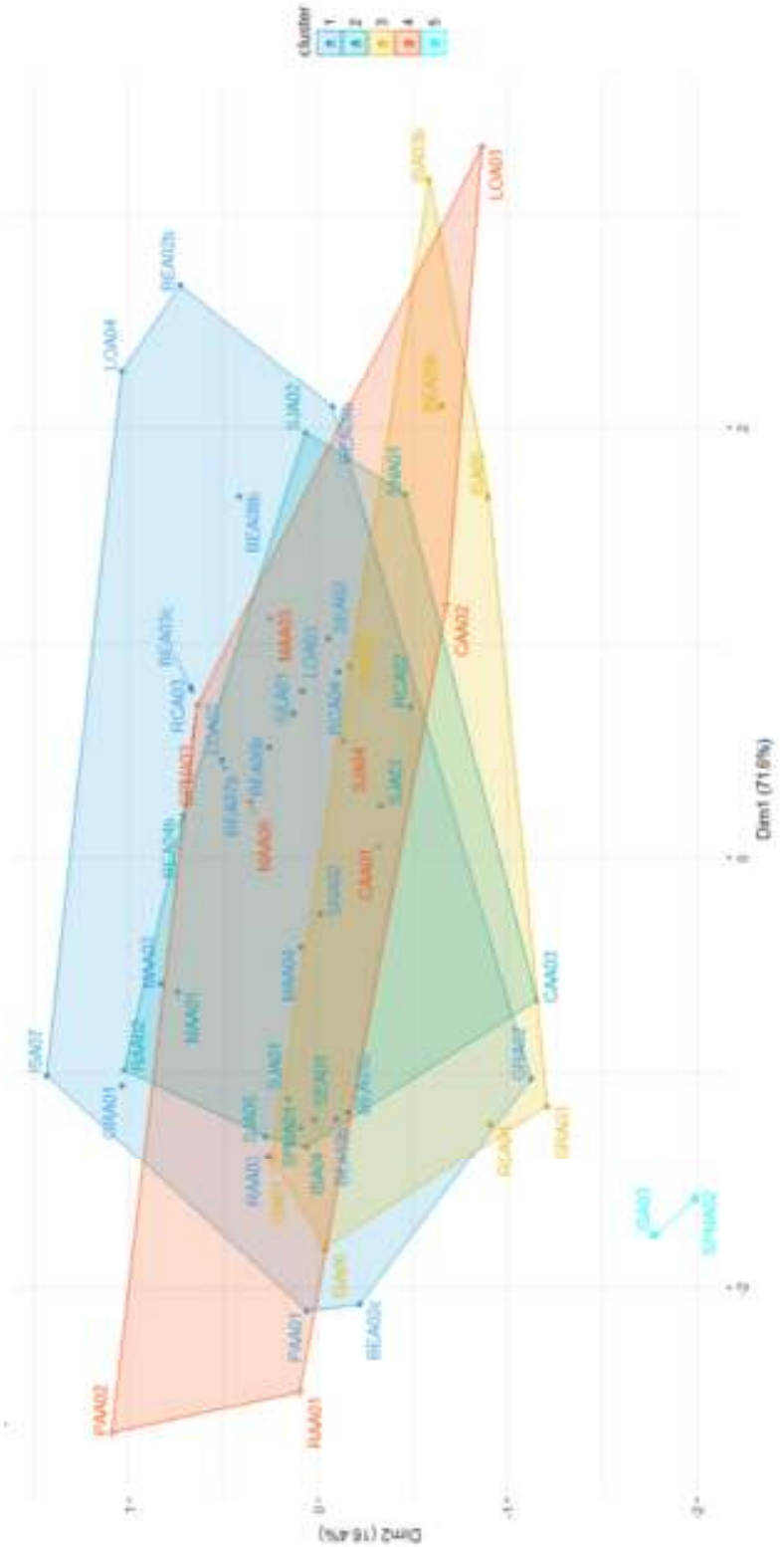

Figure 6.43. Cluster analysis of transcription levels of genes involved in CD8 T cell function in adult CSL.

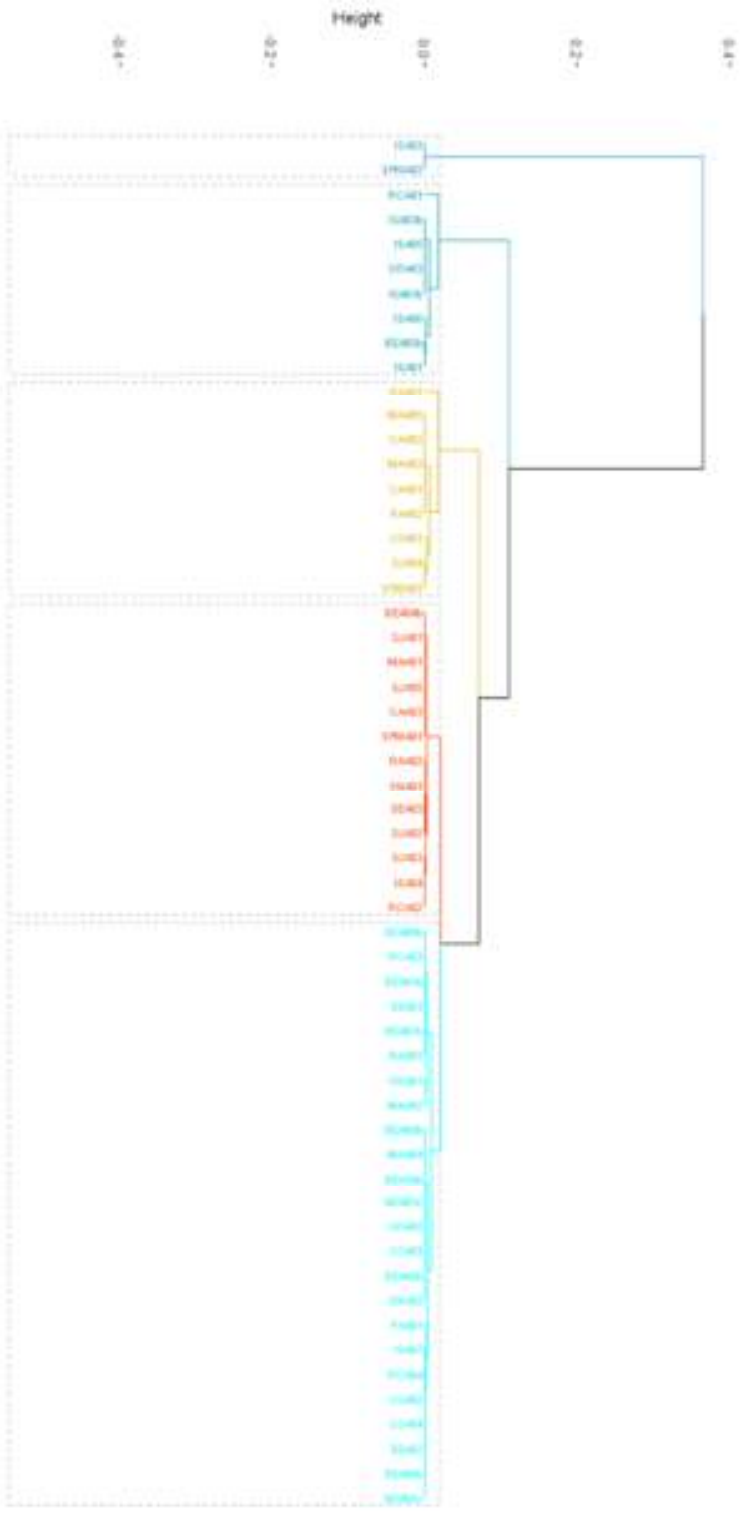

Figure 6.44 Average based dendrogram of transcription levels of genes involved in CD8 T cell function in adult CSL.

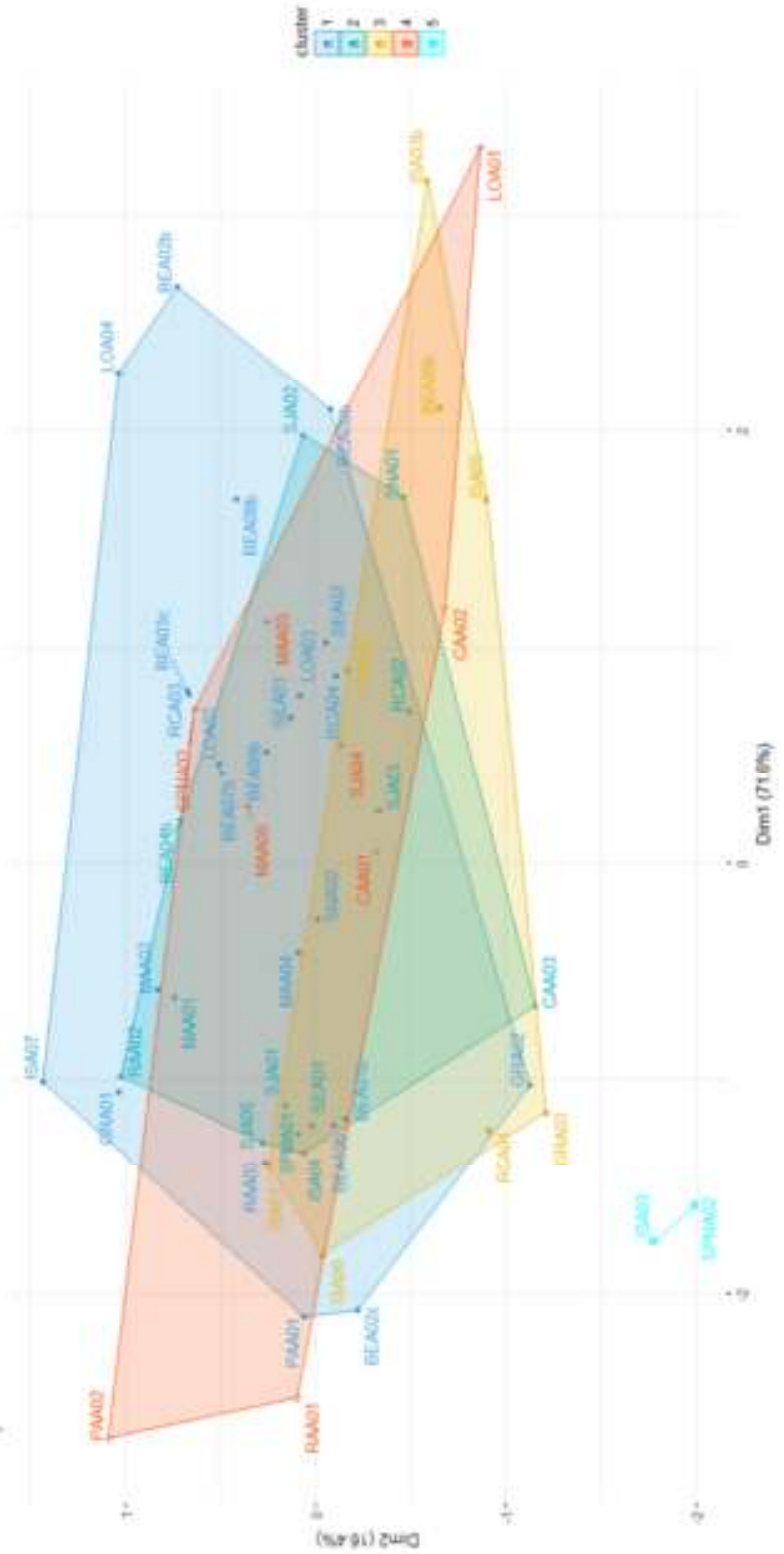

Figure 6.45 Cluster analysis of transcription levels of genes involved in CD8 T cell function in adult CSL (based on a five-cluster dendogram)..

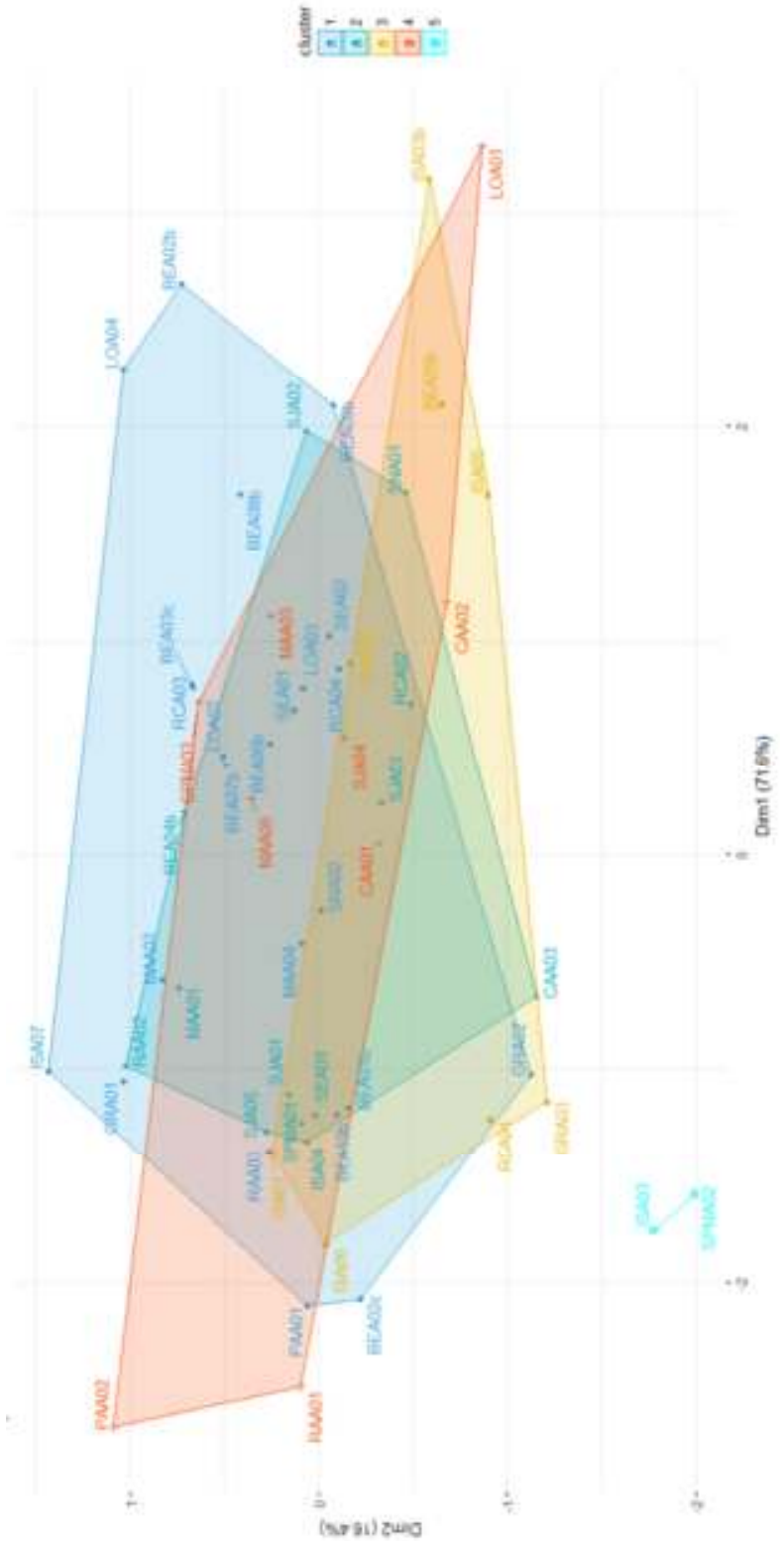

Figure 6.46 Cluster analysis of transcription levels of genes involved in CD8 T cell function in adult CSL.

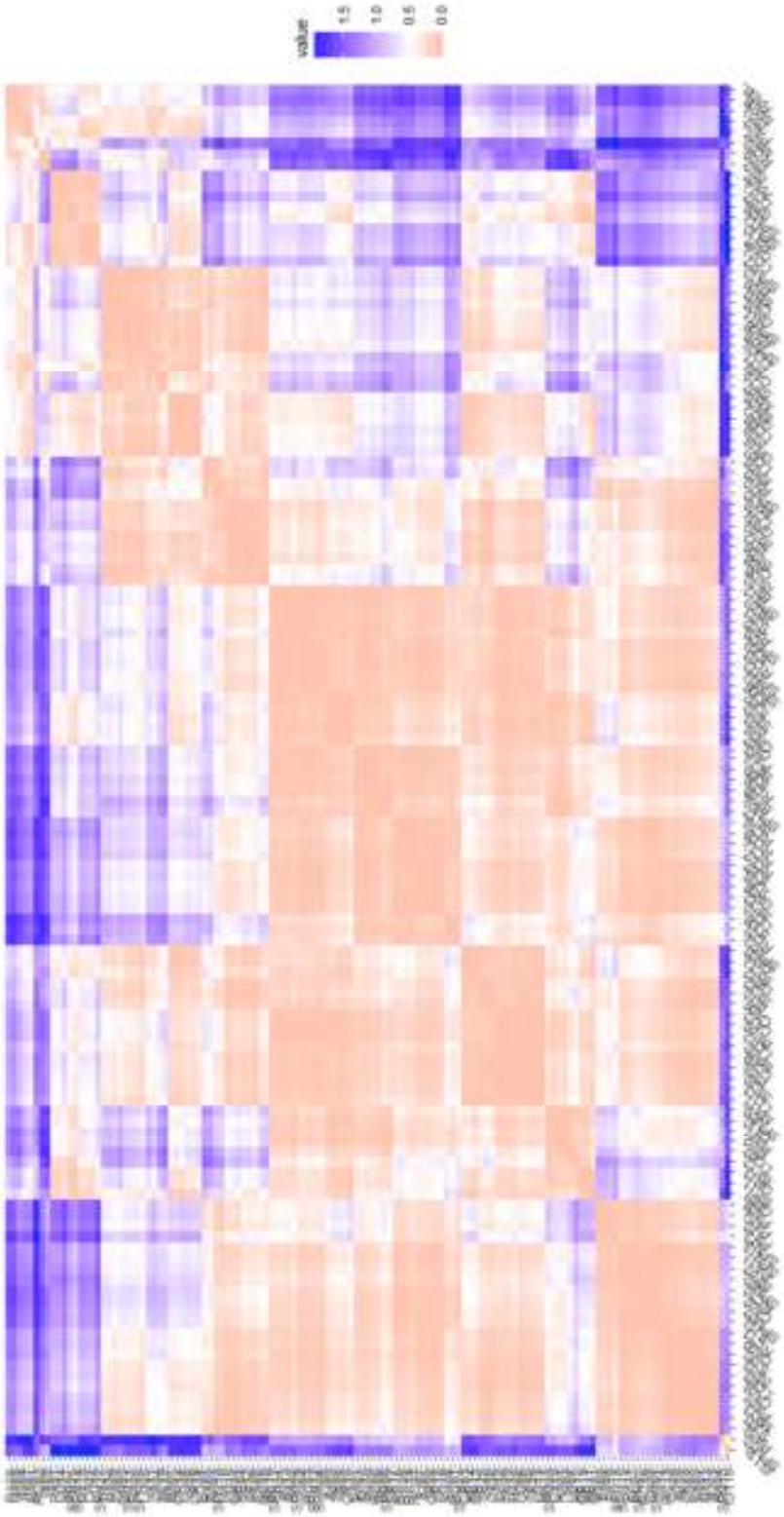

Figure 6.47 Heat map of transcriptional analysis of NK function of CSL pups.

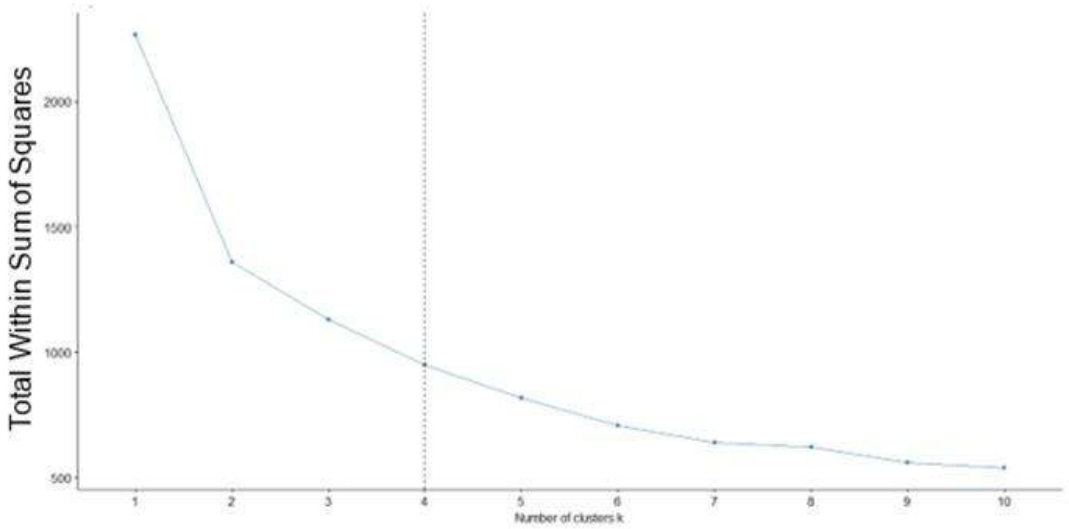

Figure 6.48 Rarefaction curve considering NK genes from pups. The dotted line shows the optimal number of clusters.

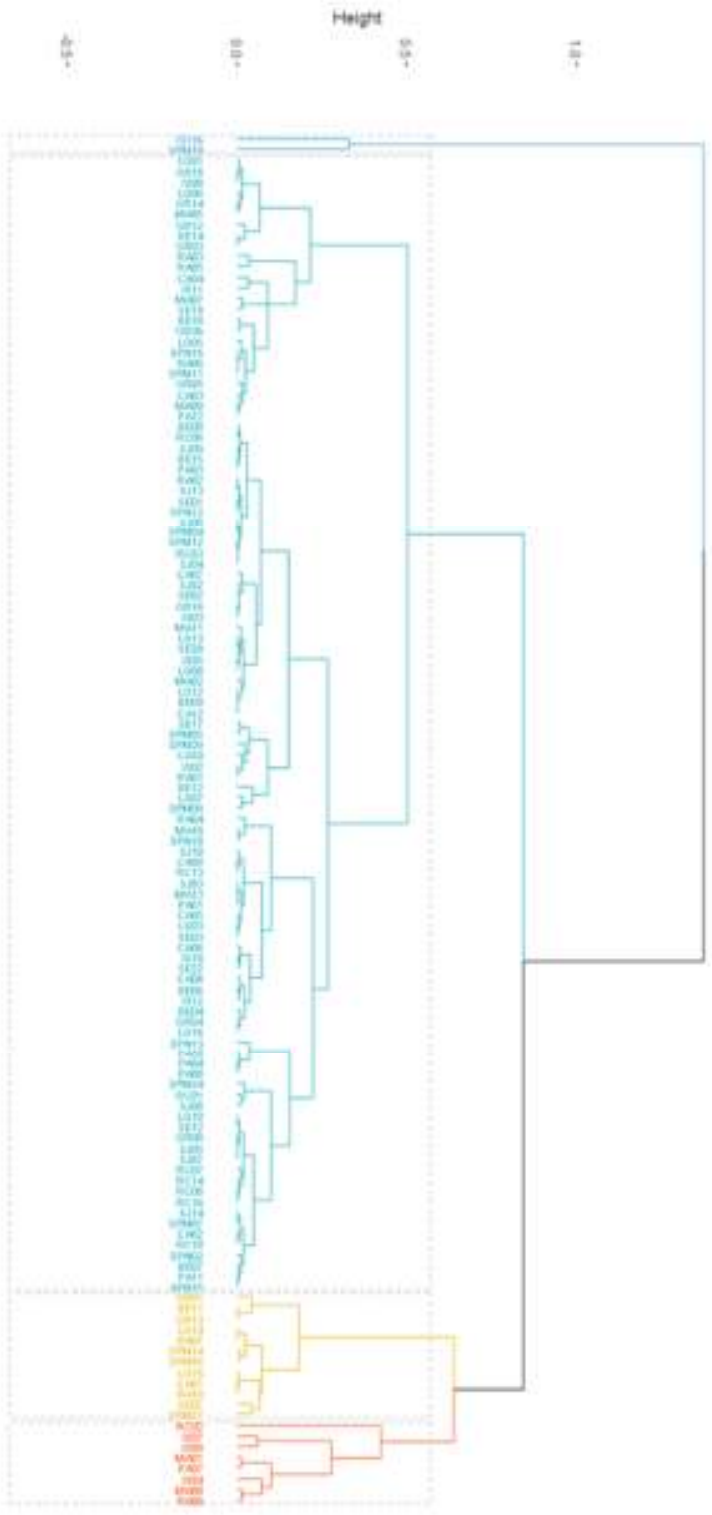

Figure 6.49. Average based dendrogram of transcriptional analysis of NK function of CSL pups.

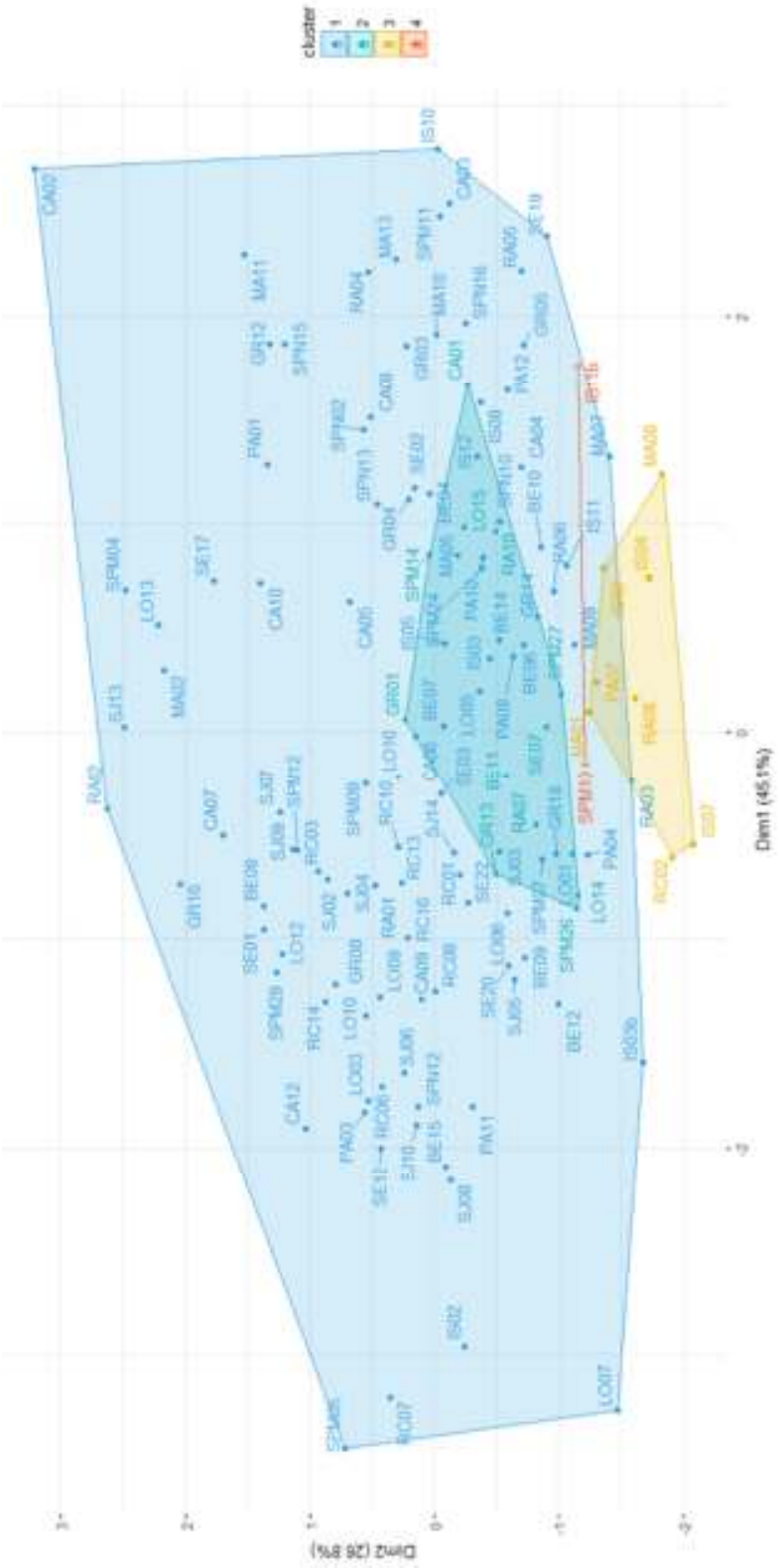

Figure 6.50 Cluster analysis of transcriptional analysis of NK function of CSL pups.

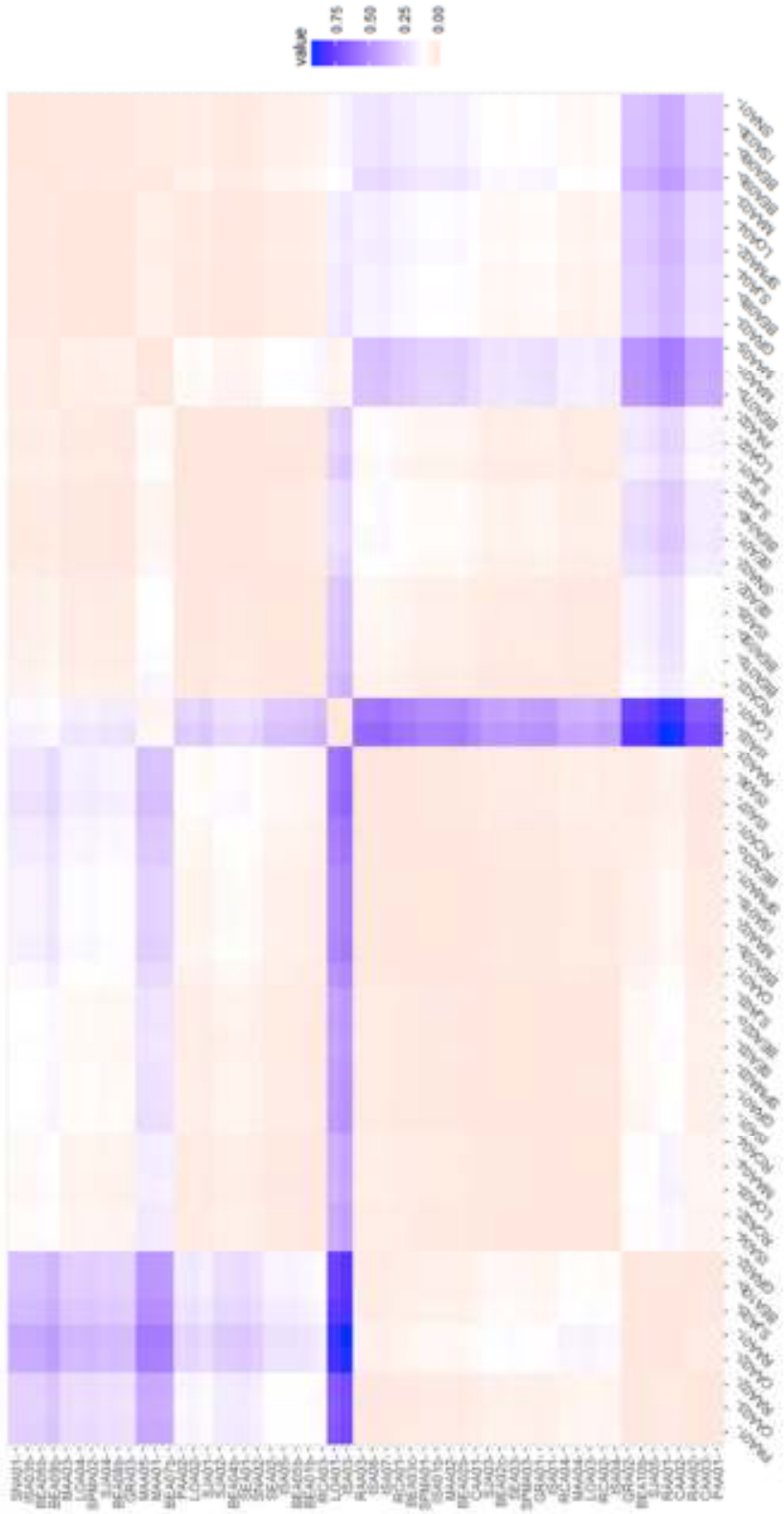

Figure 6.51 Heat map of transcriptional analysis of NK function of adult CSL.

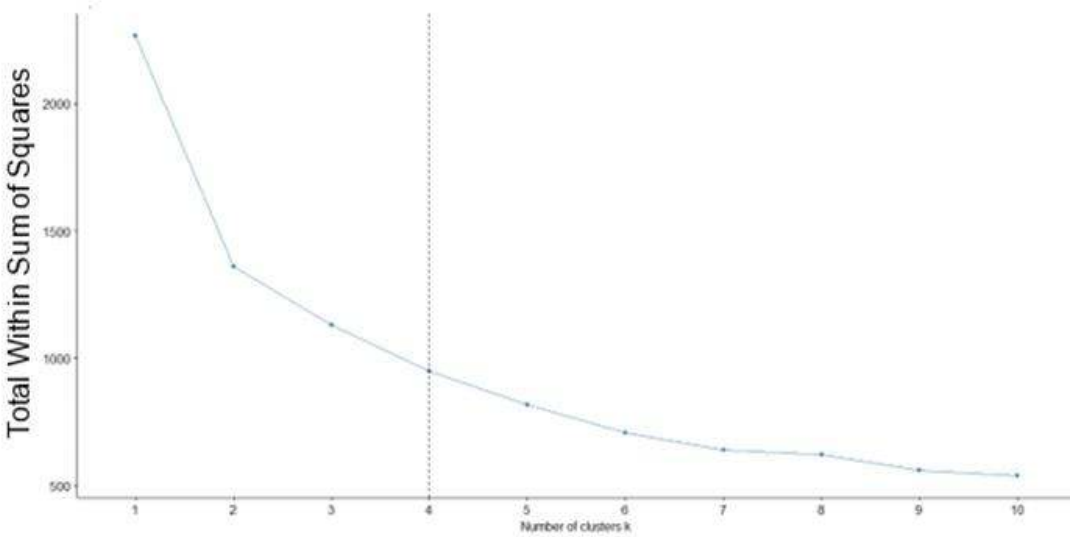

Figure 6.52 Rarefaction curve considering NK gene transcription in adult CSL. The dotted line shows the optimal number of clusters.

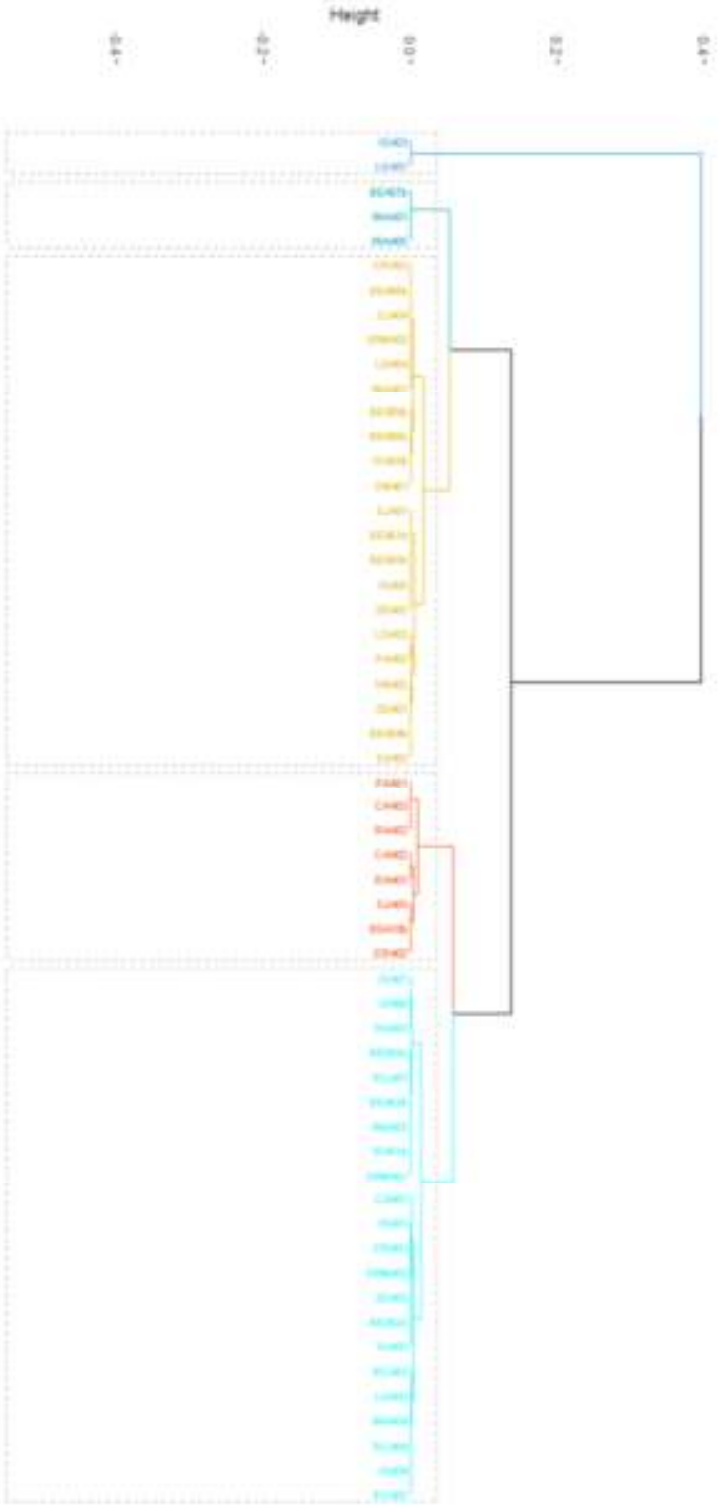

Figure 6.53 Average based dendrogram of transcriptional analysis of NK function of adult CSL.

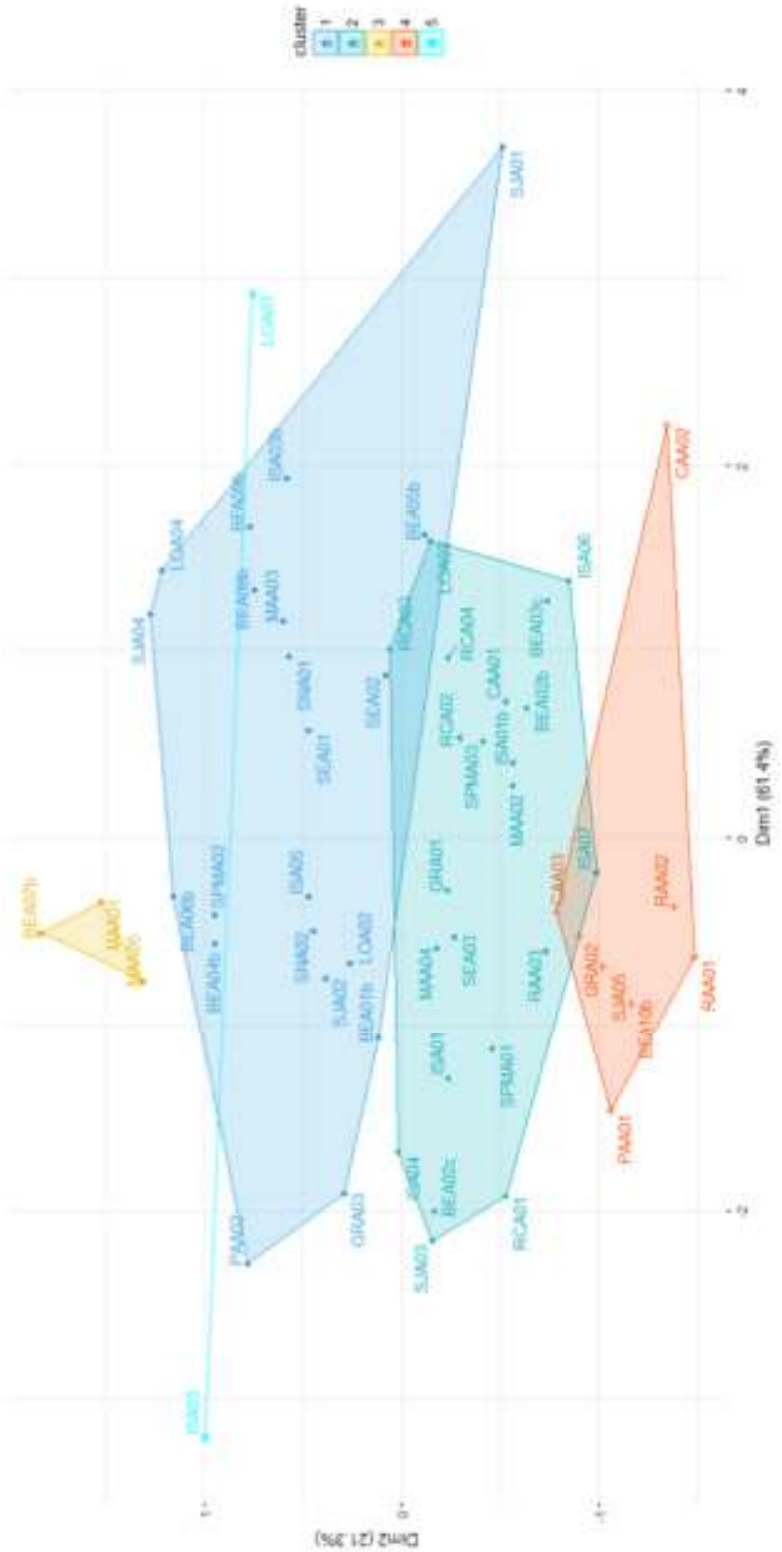

Figure 6.54 Cluster analysis of transcriptional analysis of NK function of adult CSL.

6.4 Discussion

This chapter examined spatial and age-related differences in transcription levels of key immune-related genes. Interestingly, when considered by type of immune response among colonies, I found a pattern for both Th1 and Th2 responses, represented by STAT-1 and Tbet, and GATA3, respectively. The Th1 response varied according to the age of the animals and, also showed a north-south pattern. However, when considering natural grouping, none of the clusters of genes analysed showed a latitudinal pattern. Most of the animals included in the analysis had normal immune responses. When clustering with all genes no informative pattern was observed. However, I found evidence that some individuals had an immune behavior that was distinct from the clusters.

Th1 response

Th1 responses were increased in pups compared to adults, particularly in the colonies located in the northern Gulf of California. This result was unexpected because human neonates tend to have a bias towards Th2 responses, although this pattern can change rapidly if they are exposed to microbial pathogens at birth (Berger, 2000). It is plausible that CSL from northern colonies are exposed at birth to pathogens that are not present in the central and southern colonies, or that their Th1 response is being elicited by exposure to non-pathogenic microorganisms or other innocuous factors (Grunig *et al*., 2014). Th1 and Th2 responses can increase in mice and human exposed to air pollutants (Grunig *et al*., 2014) and exposure through diet to pollutants can induce a long-term immunosuppression in pinnipeds and dogs (de Swart *et al*., 1994; Ross *et al*., 1996; Sonne *et al*., 2006).

STAT-1 and Tbet are transcription factors involved in the differentiation and maturation of T-cells (Zheng and Flavell, 1997; Decker and Kovarik, 1999; Darnell, 2002). They are involved in Th1 pro-inflammatory responses that, generally, are elicited in response to infections by intracellular parasites and during the first stages of cancer (Balkwill and Mantovani, 2001). Thus, higher transcription levels could indicate higher reactivity against pathogens or precancerous transformation. However, increased transcription could also indicate a reaction above which is needed, which could drive individuals towards an anergic status or increase their likelihood of developing autoimmune diseases.

The lower transcription levels observed in the adult females could plausibly indicate scarce prey. If resources are impoverished, the CSL would have fewer resources for energetic metabolism (Long and Nanthakumar, 2004), and could lack sufficient energy to invest in expensive immune responses, as has been reported for other otariids (Brock *et al*., 2013). It has been reported through population trends and isotopes analysis (Szteren *et al*., 2006; Aurioles-Gamboa *et al*., 2017) that the animals in the Northern have a poorer diet than those in the central and southern colonies of the Gulf of California. This can be explained due to the upwellings in the central coasts and in the midriff (Lluch-Cota, 2000), which do not allow a full admixture of the water. These animals might invest fewere resources towards expensive immune responses.

Pinnipeds appear to be able to respond to infectious challenges with complete adaptive responses even when they are very young (King *et al*., 1998; Vera-Massieu *et al*., 2015; Espinosa-de Aquino *et al*., 2017). Thus, based on the results herein observed, it is likely that CSL pups are preparing to face pathogens, and that pups from colonies in the Southern colonies face different challenges than pups from other regions. There is some evidence to support the argument that pathogenic challenges vary among colonies. For instance, *Brucella abortus* was reported in sea

lions from San Esteban (Ávalos-Téllez *et al*., 2014), and antibodies against leptospiral serovars were shown to vary significantly among colonies (Acevedo-Whitehouse *et al*, 2003b), being more prevalent in the North and Central regions, which comprise CSL colonies from Rocas Consag to San Pedro Nolasco (Avalos-Téllez *et al*., 2016). Furthermore, the nasal aerobic bacterial microbiota of CSL shifted slightly among colonies within the Gulf of California (Hernández-Castro *et al*., 2005). In the present study, natural clustering did not find a clear geographical pattern of Th1 responses for adults or for pups. However, clear groups were formed, which probably reflect individuals with strong and a low Th1 responses.

Pups from Rocas Consag were overrepresented in the strong Th1 response group. This concurs with was observed when analysing gene transcription individually, as this colony showed the highest mean for both STAT-1 and Tbet transcription levels, and it adds support to the proposed explanation that animals in this northern colony might be facing an extended immunological challenge from an early age. With the data available, it is difficult to determine whether the challenge is infectious or environmental. However, as the northernmost colony, it is likely that its proximity to the Colorado River delta increases exposeure of CSL to agricultural, industrial and domestic contaminants. On the other hand, five pups from Cantiles colony were assigned to the low Th1 response group. Interestinly, past studies have discussed the complications of assigning this colony to a specific eco-region, as it appears to behave in a different way than the group it was clustered to (Szteren and Aurioles-Gamboa, 2011). As other individuals from this colony had an average response and none was in the strong Th1 response group, it is plausible that CSL pups in this island are exhibiting some level of immunosuppression. If so, this might help explain the decreasing demographic trend of this colony (Szteren and Aurioles-Gamboa, 2011).

When analysing adults, some groups were clear, but most of the animals were assigned to the low Th1 response group. It is extremely unlikely that there is generalized immunosuppression of adult female CSL in the Gulf of California, so the most parsimonious approach is to define the unusual pattern as the high Th1 responder cluster. Here, nearly all CSL from Benitos were groped together. Considering that this colony is located in the Mexican Pacific and not in the Gulf of California, it is not surprising that the individuals from this site exhibit a different behaviour than those from other colonies. The CSL population at the Benitos Archipelago has been decreasing in the last years, and although it is currently considered stable, the increase of sea surface temperature is a cause of concern (Elorriaga-Verplancken *et al*., 2015). Furthermore, females from colonies in the Mexican Pacific reportedly have higher levels of organic pollutants than those from colonies from the Gulf of California (Ylitalo *et al*., 2005; Del Toro *et al*., 2006; Niño-Torres *et al*., 2009) and seem to be responding with high levels of Th1 activity. Although they are presumably not in lack of resources, environmental changes such as el Niño or the Blob could result in changes in infection and in higher risk of oncogenesis (see Banuet-Martínez et al. 2017).

Th2 response

When examining the Th2 response, a pattern became evident for GATA 3, considered a marker of Th2 responses (Zheng and Favell, 1997), which is involved with immunotolerance and mild responses to viruses and tumor cells (Hanahan and Coussens, 2012). Sea lions from the southern colonies as well as from Benitos had higher transcription levels of this gene, particularly the adult females, although the pattern was less marked than observed for Tbet and STAT-1. Furthermore, regardless of the colony, transcription levels were consistently higher in pups than in adult females. The pattern was especially interesting for the northern colonies, where Th1 and Th2 responses did not appear to be behaving as expected. Usually, Th1 and Th2 tend to exhibit an inverse and

polarized pattern, where one increases while the other decreases (Long and Nanthakumar, 2004; Rajesh *et al*., 2015; Romagnani, 1994; Mosmann and Coffman, 1989). The results observed in the northern colonies suggest that these CSL might have a dysfunctional immune response, although the southern regions, where responses are more differentiated in adult females, would need to be considered as the reference or normal values in order to make this hypothesis valid.

For pups, the natural clusters obtained through hierarchical analysis showed similar patterns for Cantiles, with seven pups being assigned to the low Th2 response group, while the pups from other islands grouped heterogeneously. This result was of concern, as Th1 and Th2 response appeared to be downregulated in this colony in contrast to the apparent upregulation that I found in the northern colonies. The CD4 response, when Th1 and Th2 transcripts were analysed together, did not reveal any pattern despite their explanatory power. They are not showing geographical patterns or differences by colony. Instead of that, the differences might correspond to differences in health status among CSL.

In humans, pathological states can occur when Th1 and Th2 responses are unbalanced (Pinto *et al*., 2006; Rajesh *et al*., 2015), and their simultaneous expression can increase the risk of infections and, in the long run, diminish Th1 responses (Pinto *et al*., 2006). Similarly to what I obtained for Th1, Th2 did not show differences related to geographical sources of pollution. It is unlikely that the differences in GATA3, STAT-1, and Tbet transcription levels among colonies are explained by organochlorine concentrations, as the colonies more likely to have higher levels of contaminants were not those which showed differences in the comparison among islands.

The only organic pollutant that could be producing a decrease in Th1 and Th2 responses of CSL from the Northern islands are pesticides. DDT and DDE have been reported to exceed the

ideal values in the Delta of Colorado River (Lugo-Ibarra *et al*., 2011). PCBs, however, remain low in that region, although PCB138 has been found in relatively large quantities (Lugo-Ibarra *et al*., 2011). However, other pollutants, such as mercury have sources inside the Gulf (García-Hernández *et al*., 2007; Ruelas-Inzunza *et al*., 2008) and could also be related to alterations of immune activity. Top predator fishes of a similar trophic position that were caught off the coast of Sinaloa have high levels of mercury and it is parsimonious to consider that fish-feeding predators such as the CSL can bioaccumulate this pollutant. Although in 2007 the Northern regions were not very polluted (García-Hernández *et al*., 2007) there are reports that freshwater fish that inhabit the Colorado River delta possess concentrations above the risk threshold for piscivorous mammals (Walters *et al*., 2015). *In vitro* studies performed in human cells have shown that mercury can modulate the immune system, increasing or decreasing immune responses, including lymphocyte proliferation (Shenker *et al*., 1992). Mercury is also a bioaccumulative pollutant which increases along the trophic web (Brown *et al*., 2018), so young individuals could be affected more severely than adult animals. Empirical evidence of this has, to the best of my knowledge, not been found yet.

In humans, it has been shown that maternal exposure to agricultural environments, including allergens and chemicals, can modify the balance between Th1 and Th2 response (Duramad *et al*., 2006) driving towards Th1 (pro-inflammatory) responses. Maternal exposure to organic pollutants and pesticides from agricultural environments reportedly influences $CD4^+$ responses in their infants (Duramad *et al*., 2006). If similar effects were to occur in CSL, the effects could expectedly be even more intense due to the thickness of the blubber layer of adult females, particularly because many persistent pollutants are lipophilic (Jones and De Voogt, 1999).

Cytotoxic cell populations

Eomes, granzymeB and perforin showed differences between age class and colonies, although a clear latitudinal pattern was only evident in northern colonies. For example, transcription of Eomes decreased in both adults and pups from the northern to the central colonies of the Gulf of California, but remained stable in colonies in the central and southern regions. A similar pattern was observed for granzymeB, although only for pups. In contrast, transcription levels of perforin did not exhibit a clear pattern. Together with Ly49 transcription, which remained constant, the observed pattern for granzymeB could be taken as an indication that CD8+ cells are transcribing these genes rather than other cytotoxic lymphocyte populations (Knox *et al*., 2014). The other possible explanation is that CD8+ cells are more abundant than NK cells in CSL blood. In dogs, the number of NK is thrice less than CD8+, so if this ratio is consistent in CSLs, it is unlikely that the number of CD8+ cells account the observed results. In all the colonies analysed, except for Partido and Rocas Consag, the NK and $CD8^+$ factors were higher in adult females than in pups. Th1 responses appeared to be downregulated in adults from the northern region, so the behaviour of their cytotoxic lymphocyte population in this region seems anormal.

$CD8^+$ responses appeared to be related to two variables. On one hand, there appeared to be a latitudinal trend from the northern to the Central islands (excluding San Esteban, San Pedro Mártir and San Pedro Nolasco), which could be associated with the relatively higher levels of pollutants in the Colorado River delta (García-Hernández *et al*., 2001; 2006). This can be seen in the patterns observed for Eomes, and granzymeB. It would thus appear that CSL colonies in these regions are responding to an infectious challenge (i.e. viruses) or to cellular transformation, or that their immune response is being elicited by innocuous agents, as discussed for Th1 response in pups. These populations have fewer resources than those found in the southern Gulf of California (Aurioles-Gamboa *et al*., 2017; Lluch-Cota, 2000; Szteren *et al*., 2006), so it is probable that CSL

investment in cytotoxicity will depend on the intensity of the challenge, as eliciting responses can be costly (Lochmiller and Deerenberg, 2000; Long and Nanthakumar, 2004; Brock *et al*., 2013). From San Esteban to Islotes, CSL had similar cytotoxic responses, and it is known that they have higher availability of resources than in the northern colonies. Along these populations, the immune response seems to be similar. As these islands are probably less polluted than regions close to the Colorado river as these are immediately in the river mouth, and thes islands are hundreds of kilometres from the mercury sources of Sinaloa (Ruelas-Inzunza *et al*., 2008), their immune response should be responding to infectious challenges and not to physically unrelated stimulus.

In previous studies, some pathogens have shown island related distribution and differences between Gulf of California and Pacific Ocean (Avalos-Téllez *et al*., 2016). Although antibodies against *Leptospira* were widespread (63% of prevalence), titers were generally low. This bacterium could drive the immune response in low resources years, as El Niño, but usually CSL is a non sympthomatic carrier (Avalos-Téllez *et al*., 2016). The absence of a pattern, however, concurs with other studies on different pathogens (Hernández-Castro *et al*., 2005; Soto-García, 2016), which showed non-latitudinal patterns for infection.

Natural grouping for the pup samples using these genes showed three well-defined clusters and a smaller one that overlapped the second group. These clusters, however, did not correspond to a spatial pattern as individuals of different colonies were assigned to the largest of the two clusters. It is possible that these clusters represent two different immune strategies for CD8 responses among individuals. However, three CSL from Rocas Consag were allocated to one of the small clusters, which, according to what was observed for Th1 and Th2 responses, could represent individuals that are eliciting strong immune responses (Szteren and Aurioles-Gamboa, 2011; Walters *et al*., 2015; Brown *et al*., 2018).

In contrast to pups, expression of $CD8^+$ genes in adults did not show clear clustering, but confirmed a very different responses among individuals. 16IS03A and 16SPM02A exhibited a similar response, but were quite distinct from other CSL. Although the clustering analysis did not allow inferring the meaning of these results, 16IS03A was consistently different along the analysis, and also behaved strangely, along with 16LO01A, when NK genes were analyzed. It is possible that this sea lion was facing a particular challenge, plausibly viral or oncogenic. This same scenario could help explain the similar pattern observed for 16SPM02A and 16LOA01.

Treg

FoxP3 transcription differed significantly between adults and pups but showed no obvious spatial pattern. The behavior of this gene could be due to the ontogenetic stage of the animals, as every region showed a similar pattern in transcription between pups and adult females. FoxP3 is the transcription factor that controls the formation of Treg cells (Hori *et al*., 2003). Under certain stimuli, some Treg cells decrease the expression of FoxP3, and lead to an immunotolerant status (Zhou *et al*., 2009). Lttle is known about FoxP3 in wildlife (Zhou *et al*., 2009) and even less about its ontogenetic role in pinnipeds. However, this study revealed that the relative transcription if FoxP3 is lower in adults than in pups. This was in accordance with STAT-1 and Tbet's transcription levels, so it is plausible that the immune system of adult female CSL, particularly those in the northern region, is being driven towards an immunotolerant status.

Kir and STAT6

Finally, while intriguing, the differences observed in KIR and STAT 6 transcription levels cannot be fully understood in terms of ontogenetic, oceanographic or ecological, variables as the number of samples which could be used for qPCR assays was too low. However, transcription levels of

KIR could partially support the idea proposed above that pups from the northern islands are experiencing greater immune challenges, since transcription levels were higher than in other colonies. This would concur with what was observed for Th1 response genes, Tbet and STAT-1, transcripts. KIR relative expression apparently contradicted Ly49, which remained stable among regions. However, observed differences between NK cell and Th1 transcripts support the hypothesis that changes are not a consequence of toxicity in the blood but rather are due to alterations at the organ or regulatory cell level, as was proposed for other pinnipeds (see Ross *et al*., 1996).

In contrast with what was observed when analysing results by colonies, when individuals were studied using natural groups, there were no obvious geographical pattern. The clusters were formed by samples from different islands with some individuals being excluded from the main clusters. An intermediate level of expression was most common for all of the types of response types here studied, but some CSL behaved very differently, and, in some cases, they belonged to the same colony. This could mean that when exposed to a challenge, the immune behaviour of CSL could differ among colonies. Although other data must be analysed to challenge this hypothesis, I focused on these unusual groups to find a pattern, as they could be indicative of anomalies in their immune responses.

In conclusion, natural clustering was not productive to find regionalization using immune response. However, it may allow us to determine the baseline for the immune responses in free-ranging CSL. New data can be analysed in the sight of these results and new animals can be awarded to established groups.

In sum, it is important to highlight that for both age groups, the Th1 response increased with a north-south gradient, because of their behaviour. It is possible that if pups are challenged early during their development, this particular lymphocyte subpopulation will not respond adequately when they are older. There is some evidence that could support this possibility. For instance, strong and sustained immune responses to viruses can drive to an anergic status or facilitate autoimmune diseases (Karpus *et al*., 1994). This also occurs in other types of challenges such as sustained allergies or organ transplants, which drive the immune response towards an immunotolerant status (Holt and Macaubas, 1997; Markees *et al.*, 1998; Waldmann *et al*., 2006). Furthermore, the expression patterns of Ly49 further supports this proposed explanation. The innate response represented by this gene, which is mainly expressed by NK cells (Hammond *et al*. 2009), did not exhibit any latitudinal pattern. If this lymphocyte subpopulation were being affected directly by a toxin (i.e. pollutants in the blood that act on circulating cells), NK cells would expectedly show the same pattern as the other lymphocytes. In this case, alterations of T cell expression could be mediated by alternative mechanisms, such as regulation by CD4+, macrophages or transcription processes (Glass and Saijo, 2010), which could help explain the difference in immune activity observed among adults and pups.

7. GENE TRANSCRIPTION OF CIRCULATING LYMPHOCYTES AND EPITHELIAL TRANSFORMATION OF THE GENITAL EPITHELIUM OF CSL

7.1 Introduction

The California sea lion (CSL) is a pinniped whose US population has been affected by urogenital carcinoma (Gulland *et al*., 1996; Lipscomb *et al*., 2000). This pathology has a multifactorial origin that includes genetic factors (Acevedo-Whitehouse *et al*., 2003a; Browning *et al*., 2014), infections by oncogenic pathogens (King *et al*., 2002; Johnson *et al*., 2006), and persistent organic pollutants (Ylitalo *et al*., 2005). However, none of these factors is likely to be the sole cause of urogenital carcinoma.

In other geographical regions where the CSL is also distributed, namely within the Gulf of California, and along the Pacific coast of Baja California, all the factors related to this pathology occur, but there have been no reports of this kind of cancer to date (Barragán-Vargas *et al*., 2016). It is plausible to assume that the risk factors, particularly the organochlorine pollutants, vary in terms of their intensity between regions (Barragán-Vargas *et al*., 2016), and may have their own thresholds for risk for urogenital carcinoma, as shas been reported for infectious diseases in the harbor porpoise, *Phocoena phocoena* (Jepson *et al*., 2005).

Previous work conducted on CSL from the Gulf of California reported celular transformation of the genital epithelium (Barragán-Vargas *et al*., 2016). *In situ* transformation is considered to be the first step for tumour progression, particularly for tumors related to oncogenic strains of papilloma virus (Hausen, 2002). Cellular transformation can have a benign or a malignant behavior, and the ability to metastasize is one of the last traits exhibited by malignant tumors. Prior to reaching this stage, there are various cellular pathways that can lead to a reversal of the transformation.

Cancer progression is not only dependent on cell traits. The immune system of the affected individual can foster or revert the initial steps of the cancer progression. Studies conducted in humans and laboratory animals have shown that the polarized response of M1 and M2 macrophages, or TCD4+ for Th1 or Th2 response can be predictive of the potential progression of cancer (DeNardo *et al*., 2010; Ruffell *et al*., 2010). In addition, other circulating cells are directly involved with oncovigilance and eradication of cancerous cells. Namely, TCD8+ (Chen *et al*., 2005) and NK cells (Smyth *et al*., 2005) are the cytotoxic populations that oversee cell status, and selectively eliminate those which are infected by viruses (including potentially oncogenic viruses such as OtHV-1 and papillomavirus), or that are transformed. An absence of immune activity can facilitate expansion of the tumour and invasion of other tissues (Hanahan and Weinberg, 2011).

There are other immune cells that are involved in oncosurveillance. CD4+ and Treg cells, although not directly related to the elimination of transformed cells, can direct the response of the immune system towards an antitumorogenic or protumorogenic status. CD4+ can act as Th1 or Th2, activating or inhibiting the cytotoxic action by the control of cytotoxic T cells (Beyer and Schultze, 2006; DeNardo *et al*., 2010; Ruffell *et al*., 2010). Furthemore, Treg cells, which are not directly involved in tumour control but can lead CD4 towards a Th1 (antitumorigenic) or Th2 (immunotolerant) response (Beyer and Schultze, 2006; DeNardo *et al*., 2010; Ruffell *et al*., 2010). If these immune populations decrease or dedifferentiate, the antitumorogenic activity of the body can drive towards an immunotolerant response that will in turn facilitate transformation and progression of cancer (Kim *et al*., 2007; Kim and Cantor, 2014). With this in mind, in this chapter I aimed to test whether transcription levels indicative of the activity of specific immune cell populations (Th1, Th17, CD8+, and NK) is inversely related to cellular transformation of the genital epithelium of adult CSL.

Interestingly, there are spatial differences in CSL transformation of the genital epithelium (Barragán-Vargas 2015), and taking into account that the CSL breeding colonies in the Gulf of California are distributed in genetically- and ecologically differentiated regions as has been explained earlier, it is possible that the differences in cellular transformation reflect differeces in immune gene expression patterns described in Chapter 6 of this thesis. This possibility if further strengthened by the North to South gradient in pollutant levels within the Gulf of California, directly related to Colorado's river delta spills (García-Hernández *et al*., 2006; Lugo-Ibarra *et al*., 2011). The river discharges near to Rocas Consag and Lobos CSL colonies and, to the South, its influence decreases. Marine streams form a natural barrier in the midrif, which does not allow complete admixture of water between the North and South of the Gulf of California (Lluch-Cota, 2000). Upwellings from the Pacific Ocean, which are supposed to have lower concentration of pollutants, as they come from less anthropized sources, contribute to this barrier and also lead to differences in the coast of Sinaloa. Some pollutants, such as as PCBs and DDT, are known to cause cell transformation directly (Robertson and Ludewig, 2011). However, the concentrations required for this action far exceed reported concentrations in wild CSL (Ylitalo *et al.,* 2005; del Toro *et al.* 2006). Thus, it is likely that the effect, of exposure to environmental organochlorines could lead towards immunomodulation and less than adequate immune responses, which in turn could impact CSL outcome if infected by potentially oncogenic pathogens.

7.2 Methods

7.2.1 Sample collection

Samples of peripheral blood were obtained from 52 adult females captured in the summer (july) of 2014 and 2016 in colonies from the Gulf of California and Mexican Pacific. Cervical cell swabs had been collected for 51 of these individuals as part of a larger study on sea lion health, and the data on cytology was available to me. The protocol for blood collection and separation of the buffy coat was described in Chapter 6, and the protocol for cervical swabbing has been reported elsewhere (Barragán-Vargas *et al.,* 2016). Briefly, sterile cytological brushes were gently inserted to the cervix, which had been exposed by using a sterile speculum. The brush was smeared on a glass slide, fixed immediately with Cytofix, and stained in the lab using a modified Papanicolau stain (Barragán-Vargas *et al.,* 2016).

7.2.2 Cytology and Histology analysis

All cytological analyses of the PAP smears had been carried out by Cecilia Barragán-Vargas, and the data was kindly provided for this study. The data included the Bethesda classification for the cervical smears for each CSL, namely I considered squamous cell atypia (ASC), atypical squamous cells of indetermined significance (ASCUS) and low grade squamous intraepithelial lesions. Also, for each slide there was data on the quantity of koilocytes, binucleated cells, reactive cells, neutrophils, and lymphocytes, which had been normalized to smear surface for each slide (see Barragán-Vargas et al., 2016).

7.2.3 Gene expression data

As part of my thesis I had performed gene-expression quantitation analyses to assess the activity of specific immune cell populations of the buffy coat of pups and adult CSL (see details in Chapter 6). For this Chapter, I used the data on gene transcription collected for the adult females. I had transcriptional profiles that were representative of CD4+ Th1 (STAT-1, Tbet) and CD8+ Th1 (EOMES, perforin, and granzymeB) responses, Th2 responses (GATA3), Treg responses (FoxP3), and innate cytotoxic response (Ly49, perforine, and granzymeB).

7.2.4 Statistical analysis

I examined the relationship among the different cell types (koilocytes, reactive cells, binucleated cells, neutrophils, and lymphocytes) and the level of transcription of each gene, and included region, colony, and year of sampling as variables. I built a correlation table in R studio (Vienna, Austria), and used a Mantel algorithm for all data and for gene clusters. Additionally, I tested the correlations gene by gene and by gene clusters for all the transformed phenotypes.

Generalized linear models (GLMs) were used to determine the relationship between genes and the quantity of each of the epithelial cell types. The relationship between the presence of genes and the number of each of the cell types was examined by independent GLMs that included colony as an explanatory covariate. The distribution of the data was examined using *data2fit* in the *rriskDistribution* package (Natalia Belgorodski, 2017).

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7.3 Results

Neutrophil counts conformed to a log-normal distribution, and were consequently log-normalized for analysis. The other cell types, based on their q-q plots, seemed to fit a negative binomial distribution and were thus transformed to a 0 to 1 scale before defining the model family.

There was no difference in the number of binucleated epithelial cells (GLM; $F_{34,23}$=0.8102, p=0.6304), reactive cells (GLM; $F_{34,23}$= 1.7563, p=0.1229), koilocytes (GLM; $F_{34,23}$=0.6079, p=0.8032), and neutrophils (GLM; $F_{34,23}$= 1.1642, p=0.3622) among colonies. However, lymphocytes varied significantly (GLM; $F_{34,23}$=2.9766 p=0.01318), being highest for adult CSL from San Pedro Martir and San Pedro Nolsaco colonies (Fig. 7.2).

When using all of the data together, the Mantel comparison showed no pattern of correlation among genes and any of the cellular phenotypes (Mantel statistic r =- -0.03943, significance = 0.605). This was also the case when only considering Th1-related genes (STAT-1 and Tbet), genes expressed by CD4 cells (STAT-1, Tbet, GATA3), or cytotoxicity-related genes (Eomes, Ly49, perforin and granzymeB) (r = -0.0532, significance =0.664; r = -0.05272, significance =0.631 and r = -0.07327, significance = 0.784, respectively). Individual correlation values among genes and cell counts are shown in Table 7.1. None of the correlations were statistically significant.

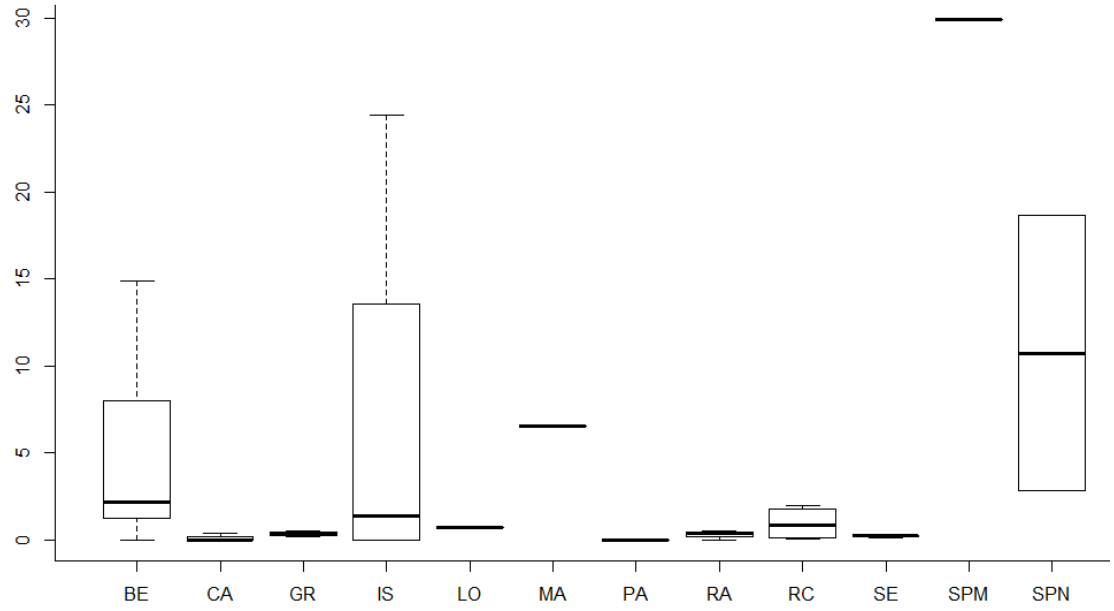

Figure 7.1 Lymphocyte counts in the cervical smears of adult CSL from different colonies in the Gulf of California.

Table 4. Correlation values among individual gene transcription levels and epitelial cell phenotypes. p-values are shown below the correlation values.

	Koilocytes	Binucleated	Reactive	Neutrophil	Lymphocyte
Eomes	0.1695 0.3302	0.0438 0.8025	0.1918 0.2696	0.0296 0.8657	-0.0094 0.9572
FoxP3	0.3215 0.0889	0.0366 0.8504	0.2274 0.2355	-0.0132 0.9456	0.1452 0.4521
GATA3	0.0947 0.5999	0.0687 0.7037	0.0094 0.9582	-0.0331 0.8547	0.2612 0.1419
GranzymeB	- 0.0381 0.8277	0.0292 0.8675	- 0.1451 0.4054	0.2534 0.1419	0.0940 0.5909
Ly49	- 0.0791 0.6614	- 0.1686 0.3482	- 0.0272 0.8805	0.1178 0.5138	- 0.0220 0.903
Perforin	0.1078 0.5438	0.02550 0.8862	0.0550 0.757	0.0363 0.8382	- 0.1724 0.3294
STAT-1	-0.2031 0.2569	0.1103 0.5409	- 0.1743 0.332	-0.2151 0.2293	0.0564 0.7549
Tbet	0.0412 0.8196	0.1155 0.5221	-0.0459 0.7995	-0.0232 0.8979	-0.1848 0.3031

7.4 Discussion

The rising incidence in CSL urogenital carcinoma has sparked concern about its causes. There are as yet no reports of this pathology in Mexican populations, although pre-cancerous transformation of the genital epithelium of CSL has been reported in the Gulf of Califonria (Barragán-Vargas *et al*., 2016). Sea lions in this region are exposed to the same factors as those animals in Pacific US coast (Maldonado *et al*., 1995; Ylitalo *et al*., 2005; Bowen *et al*., 2006; Del Toro *et al*., 2006; Niño-Torres *et al*., 2009). Thus, the origin of urogenital carcinoma is likely due to quantitative and not to qualitative differences. It is plausible that, if same factors are involved, there are transformed

cells in Mexican CSL populations that are resolved before the start of cancer (Barragán-Vargas *et al*., 2016).

Variations in counts of genital cell types were examined by colony, and in general values remained stable across colonies. Only CSL from San Pedro Martir and San Pedro Nolasco showed high levels of lymphocytes. Regretfully, the tissue sample was not suitable for cytological analysis due to improper fixation of the slide. Infiltration of lymphocytes in cervix cancer has been studied for long time (Nagell *et al*., 1978). It usually is associated with a good prognosis, and lower risk of metastases (Nagell *et al*., 1978; Evans *et al*., 1997). Infiltrated lymphocytes can respond to virus infection in the genital tract (Evans *et al*., 1997). This concurs with the fact that this sea lion was infected with OtHV-1 (Cecilia Barragán-Vargas, unpublished data).

It was also interesting that sea lions from El Partido colony appeared to have a higher number of metaplastic cells, although statistically significance was not reached with the number of samples available (the p-value was below 0.1 but remained above 0.05). El Partido is localized in the central rift of the Gulf of California. The population in this island was decreasing in 2006 (Szteren et al. 2006) and according with a 2011 report from the National Park Commision of Mexico (CONAMP), sea lion body condition in this colony was one of the lowest in the Gulf of California.

Metaplastic cells have not been considered indicative of malignancy for several decades (Fluhmann, 1953), although it is recognized that they can be indicative of altered estrogen receptor activity (Risbridger et al., 2001). In the California sea lion, an increase in the number of estrogen receptors has been related to metastasis of urogenital carcinoma (Colegrove et al., 2009) and the organochlorines considered in this thesis are known to mimic estrogen (Robertson and Hansen 2015). Thus, although the presence of metaplastic cells does not imply malignant cellular

transformation, both can be related to variations in the expression of estrogen receptors. Considering that El Partido sea lion colony has a moderate to high risk of extinction (Szteren et al., 2006) and, that the colony behaves differently to most colonies in many ecological analyses, it would be pertinent to conduct long terms surveillance of the behavior of the genital epithelium of sea lions from this colony, as metaplastic cells could potentially be indicative of aspects of health and exposure to contaminants.

Various blood biomarkers of cancer are commonly measured in human clinical medicine and are usually considered prognostic at early stages (Palmer *et al*., 2008). In free-ranging animals, such as the CSL, similar biomarkers have not been validated. In this thesis I selected specific genes based on their mechanistic relevance, rather than their diagnostic relevance. However, none of the transcription levels of the selected genes were clearly related to the transformation status of the genital tract. It is probable that the lack of association is due to the fact that CD4+ and CD8+ cells are not usually tumor-infiltrating cells (Engelhardt *et al*., 2012). Cases of human cervical cancer, which are histologically similar to the CSL urogenital carcinoma (Colegrove *et al*., 2009), have shown a weak correlation between tumor development and the presence of these cells *in situ* (Jaafar *et al*., 2009). However, there was a suggestion that transcription levels of FoxP3, which is expressed by Treg cells, was related to the number of koilocytes. FoxP3 infiltrated cells are common in human papillomavirus infections (Jaafar *et al*., 2009). Higher transcription levels are correlated with bad prognosis, including cancer and metastases and, additionally, human papillomavirus infection also correlates with the presence of koilocytosis (Jaafar *et al*., 2009). Future studies should aim to increase the sample size to assess the significance of the suggested trend between FoxP3 expression and the number of koilocytes in the California sea lion.

This Chapter constitutes a first attempt to understand the relevance of selected immune effectors for cervical epithelial transformation, and to identify potential biomarkers of cervical transformation in adult female CSL. My results showed that transcripts from circulating lymphocytes are not good markers of epithelial transformation of the genital tract. However, Treg activity showed interesting and promising trends. In humans, Treg cell activity is related to a bad prognosis of cancer, and has been associated with koilocytes (Jaafar et al. 2009), which are markers of cancerous transformation. Further studies should aim to examine other markers of Treg cell activity as they could be useful to detect early stages of urogenital cancer in free-ranging CSLs.

8. General discussion

California sea lions (CSL) have been affected by urogenital carcinoma for the past three decades (Gulland *et al*., 1996). During this period, various studies have identified some of the risk factors related to it, including viruses and organic pollutants (Browning *et al*., 2015). The main aim of my thesis was to examine the activity of CSL oncogenic and antiviral immune effectors under experimental *in vitro* conditions that mimicked environmental levels of common PCB congeners. In addition, I measured the transcriptional levels of key immune genes expressed by circulating lymphocytes in free-ranging, apparently healthy CSL pups and adult females from the Gulf of California. To date, most of the research on urogenital carcinoma has focused on sea lions from California, where the urogenital carcinoma has been reported. By analysing CSL from non-affected populations, namely those from the Pacific coast of Baja California and Gulf of California, I was able to gain some insight about the activity of immune effectors that could be important for

the early development and the control, or reversal, of cell transformation in the CSL genital epithelium.

Unexpectedly, immune cell activity was not decreased by exposure to PCBs at environmentally-relevant levels. On the contrary, proliferation increased when exposed to low levels of non-dioxin like PCBs. This concurs with what has been reported for other pinnipeds (Mori *et al*., 2006). However, higher PCB concentrations, seemed to revert this trend. Furthermore, innate cytotoxicity against cancer cells, which had not been tested previously, increased when exposed to two non-dioxin like congeners. Although the extrapolation of results conducted *in vitro* experiments to natural conditions should be done with caution, owing to the many interactions between different physiological components that could increase or decrease these effects, it was interesting to observe that the previously reported levels of organic pollutants (Ylitalo *et al*., 2005; Del Toro *et al*., 2006; Niño-Torres *et al*., 2009; 2010) did impact lymphocyte proliferation and NK-cell like activity of CSL. In particular, having found that exposure to pollutants can increase immune activity is worrying, because a constant or long-lasting immune stimulus can drive the system to not able to respond when required (Karpus *et al*., 1994). It is possible that CSL exposed to high levels of PCBs throughout their lives might be at a higher risk of developing cancer due to suboptimal antiviral and oncovigillant immune responses (see Kakuschke and Prange, 2007).

Several interesting patterns emerged from the analysis of transcription levels. First, the differences in immune activity between pups and adults could obey to ontogenetic factors as well as to environmental factors. When analyzing spatial differences in transcriptional patterns, I had predicted that sea lions from colonies that were nearer to more industrialized lands would show signs of more moderate immune activity and, due to that, these animals could be more inclined to infections and epithelial transformation. I did not find clearcut evidence that this is the case.

However, there were some inter-colony and inter-regional differences among colonies, for both age groups, which are most likely explained by extrinsic factors, as genetic homogeneity has been determined for CSL in this area (Maldonado *et al*., 1995). Identifying these factors is the key issue, and here I will attempt to discuss potential factors.

Sudden exposure to high levels of persistent organic pollutants may affect circulating immune cells directly, similarly to what I recorded *in vitro*. However, these pollutants do not remain high in the blood, owing to their lipophilic properties (Jones and De Voogt, 1999). Thus, high levels of pollutants in the blood would only be likely to occur during specific events. First, sudden exposure to high levels of organic pollutants spilled into the environment. However, there has been no evidence of industrial and agricultural spills in the region. DDT levels have not increased since 70's studies (Le Boeuf, 1971; Delong *et al*., 1973, Gilmartin, 1976) and PCB congeners detected in sea lion and fin whale blubber are those that are more stable and persistent, while less chlorinated congeners are less abundant (Niño-Torres *et al*., 2009, 2010), implying that organochlorines are at least stable in the Gulf of California.

Pups receive high amounts of PCBs via nursing, as maternal blubber lipids are mobilized to form milk. Mothers discharge high levels of pollutants to their pup, especially to first borns (Addison and Brodie, 1977; Beckmen *et al*., 1999; Donohue *et al*., 2002). Thus, the pups' immune system is subjected to a great challenge (Beckmen *et al*., 1999) during a critical step of development and immune maturation (Espinosa de Aquino *et al*., 2017), which as a consequence can impede the correct development of the immune system. Studies conducted in mice and seals have shown that lymphoid organs, such as the thymus, of young individuals can become atrophied when exposed to dioxin-like compounds with consequences once the animal is adult (Holladay and Smialowicz, 2000; Beineke *et al*., 2005). Conceivably, effects would be even more severe, if

circulating immune cells are constantly exposed to high pollutant levels during events of lipid mobilization (Peterson *et al*., 2014).

In California, it seems that most of the cases of metastatic urogenital carcinoma occur in young sexually mature adult males, although this could be biased due to the age class of animals that commonly strand (Gulland et al. 1996; Demming *et al*., 2018). It is likely that the increased risk is due to males being unable to lower their pollutant levels by transferring them to pups, as adult females do. However, when males begin their sexual life, they will undergo a period of limited or null food consumption for the first time, thus suddenly receiving PCBs in the blood following to lipid catabolism. Females also fast, at least partially, during nursing, and invest a large proportion of their resources to produce milk. This would, theoretically, expose them to the same challenge as males. But, the offload of lipophilic pollutants during lactation reduces their exposure to PCBs throughout their reproductive life. Males are unable to lower pollutant levels by transferring them to the pups. If sexually-mature males can surpass this critical stage and face the cellular transformation with a mature immune system, they would likely reduce their risk of developing urogenital carcinoma. Although it is possible that cell transformation still occurs after this period, there are few older stranded animals that have carcinoma (Greig et al., 2005). I propose that during the first years of reproductive life, the immune system of male adults is "trained" due to repeated cycles of starvation – PCB exposure – immune modulation – cell transformation, and, if they are successful, the might become more effective in the elimination transformed cells.

Of course, in addition to these mechanisms related to the species' reproductive physiology, juvenile, subadult, and adult males and females alike could be similarly affected during climatic events, such as ENSO or The Blob, that lead to decreased prey availability, and starvation. This could facilitate PCB mobilization due to depletion of fat energy stores. If this were to occur, a

decrease in the immune activity of circulating cells due to high levels of PCBs could increase the likelihood of infections. However, in terms of cancer, progression of *in situ* malignant transformation is likely to require a longer-lasting suppression of the immune system.

The mononuclear cells I used for the *in vitro* experiments had been obtained from adult sea lions. If we consider the reported effects of PCBs in various species, immune responses are likely to be more exaggerated in pups since immunomodulation is not as effective at this stage (Basha *et al*., 2014). Infection by OtHV-1 appears to be common in the genital epithelium of pups, and there has been evidence of pre-cancerous transformation of the epithelium (Barragán-Vargas *et* al., 2016), although no cases of urogenital cancer have been described in this age group so far (Demming *et* al., 2018). It is possible that the overly active immune system of pups helps to stop the development of carcinoma in young animals. However, if the pups are sustainedly exposed to high pollutant levels, an over-active immune system could be problematic when they reach adulthood, as they could easily become suboptimal responses, or lead to anergic states, and these vulnerable adults would easily contract disease following exposure to infectious agents or reduce their oncovigilant immune activity.

To conclude, taking into account the California sea lion's susceptibility to carcinoma, a cellular pathology that is resolved or contained by a healthy immune system, and the relationship between the immune effectors and high levels of organic contaminants, the role of the species as an ecosystem sentinel is reinforced. To date, urogenital carcinoma has only been reported along the California coast, but if the proposed anthropic character of this pathology is confirmed, it is our responsibility as scientist to use our knowledge to monitor key risk factors and inform policy makers to help prevent its expansion. One of the objectives for future studies could be to create a predictive model for the pollutant threshold to identify those populations under risk of suboptimal

immune responses, anergy or immunosuppression. Particular attention should be paid to understanding pup immune responses, as this age class appears to be more vulnerable, and immune insults can drive to anergic states in adults.

In addition to the ontogeny of CSL and the character of the persistent organic pollutants, it is important to increase our knowledge of the cancerous tumor itself. Several of the animals I analyzed here had precancerous lesions, and four females had proliferation of the vulvar epithelium (Barragán-Vargas, data not published), although none of them showed evidence of urogenital carcinoma. This suggests that even facing the same risk factors such as pollutants and oncogenic viruses, the end result will depend on the immune status of an individual.

Future perspectives

To monitor the CSL population, we need to determine pollutant concentrations along the coast and find robust indicators to monitor the behavior of these contaminants along different colonies. If a pollutant threshold can be established and is used for monitoring sea lion populations in regions currently unaffected by urogenital carcinoma, it would be possible to detect risks early enough to implement conservation and management strategies that could help to mitigate the risks. It would be important to check for even minimal variations in pollutant levels, particularly in pups, as excessive immune stimulation can lead to suboptimal adult immunity. An analysis of lymph node transcripts of dead pups, together with the determination of their blubber organic pollutant profile, could complement our results.

9. MAIN CONCLUSIONS

The main conclusions reached by this thesis are:

- Lymph nodes are a good source of lymphocytes that are viable after being maintained frozen following collection for at least one month. Conducting in vitro assays can help provide information to understand complex relationships between immune effectors and organochlorines, and can avoid heterogeneity among samples. This allows consistent laboratory results to be challenged in the field.
- Non-dioxin like PCBs are the main immuomodulatory elements among the congeners tested. CSL appear to behave differently to traditional laboratory models and, due to that, extrapolations have to be avoided as much as possible.
- The immune system of adult CSL in the Gulf of California does not reflect latitudinal patterns. Instead of that, interindividual differences appear to be more important that interregional or intercolony differences. This does not discard the possibility that exposure to organochlorines is regulating the immune response.
- There are differences in expression of immune transcripts, particularly those related to Th1 responses, between pups and adults. Pups exert a stronger antitumorogenic response in the same conditions. Except for sexually-transmitted pathogens, animals in the same region would presumably be exposed to the same pathogenic challenges. It is likely that my results are indicative of ontogenetic constraints of oncosurveillant immune effectors in this species.

- CSL from El Partido showed lower Th1 response than the other colonies. Together with the studies on its population trends, this colony seems to be particularly vulnerable to environmental factors that could impact their immune system.
- CSL from the Gulf of California appear to be, at least for now, not prone to develop urogenital carcinoma. However, they are still vulnerable to changes in their environment that could impact oncovigillant immune effectors. Although there is not a normality reference, some of its populations showed concerning patterns in their immune response. The occurrence of atypical climatic phenomena could disrupt the normal trends, particularly of individuals in the more vulnerable islands.

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11. APPENDIX I

qPCR standardization

For qPCR, all genes but KIR, G3P and RP519, amplified at 55-57ºC. KIR amplified at 61-63ºC but was not standardize as none of the reference genes amplified at this temperature. New KIR primer were designed and reordered and they also amplified at 55 degrees. G3P was discarded as a reference gene and RPS5 and HpRT were selected. Curves for all selected genes are shown: Ly49 (Fig. A1) FoxP3 (Fig. A2), GATA3 (Fig. A3), Tbet (Fig. A4), STAT1 (Fig. A5), STAT6 (Fig. A6), GranzymeB (Fig. A7), Perforine (Fig. A8), KIR (Fig. A9) HpRT (Fig. A10), and RPS5 (Fig. A11)

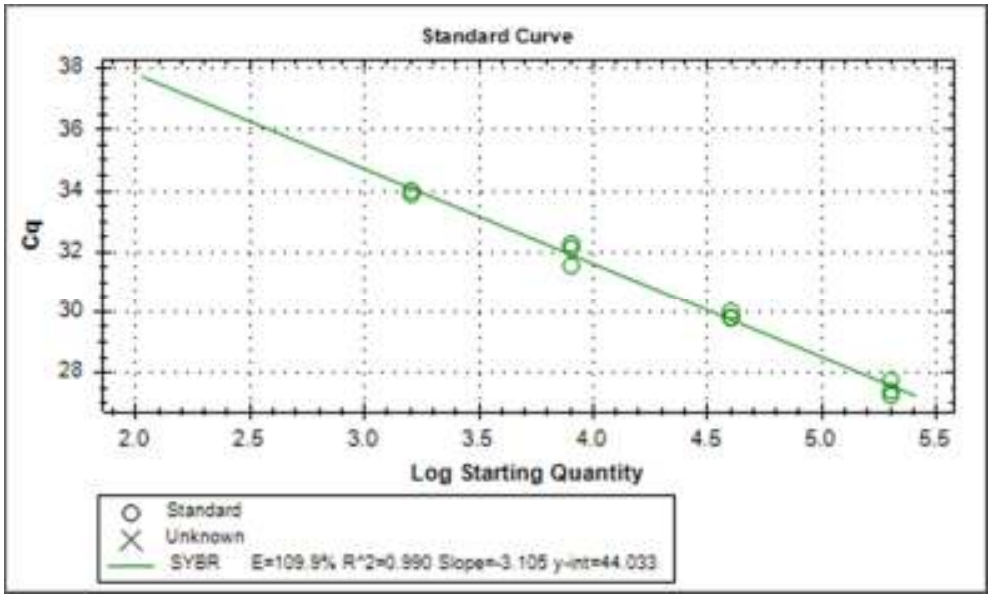

Figure A1. Standardization curve for Ly49.

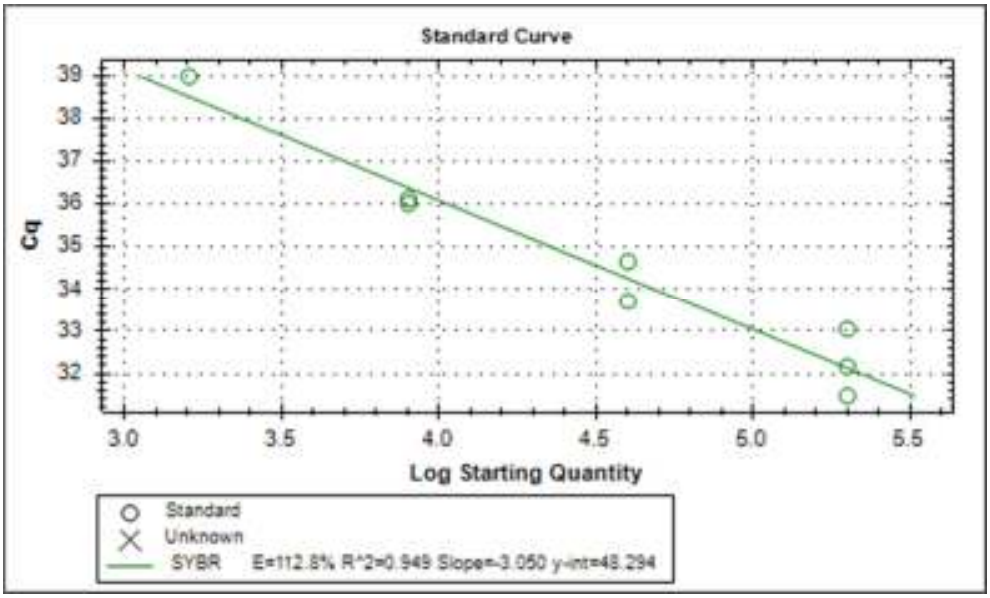

Figure A2. Standardization curve for FoxP3.

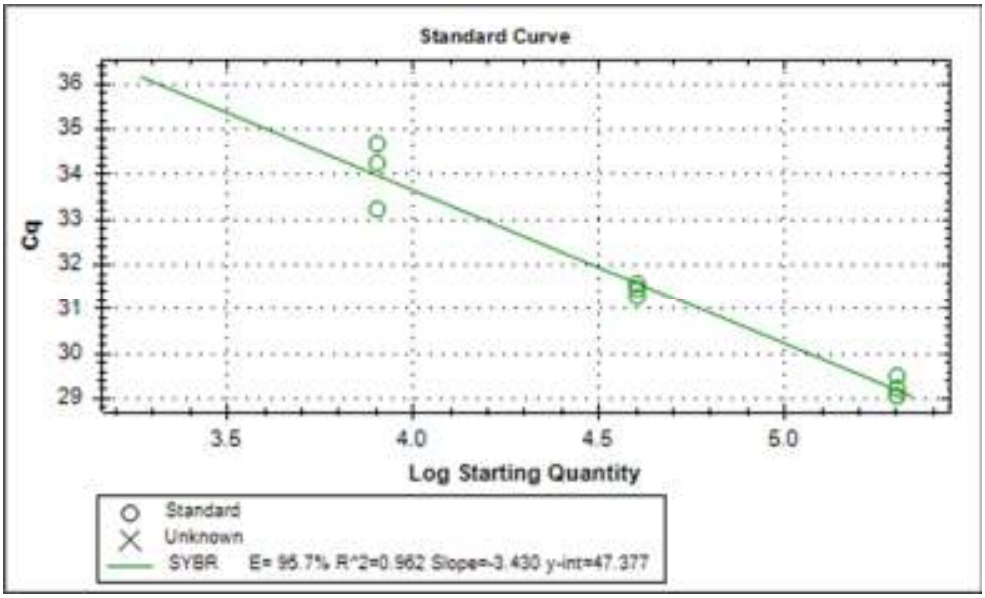

Figure A3. Standardization curve for GATA3.

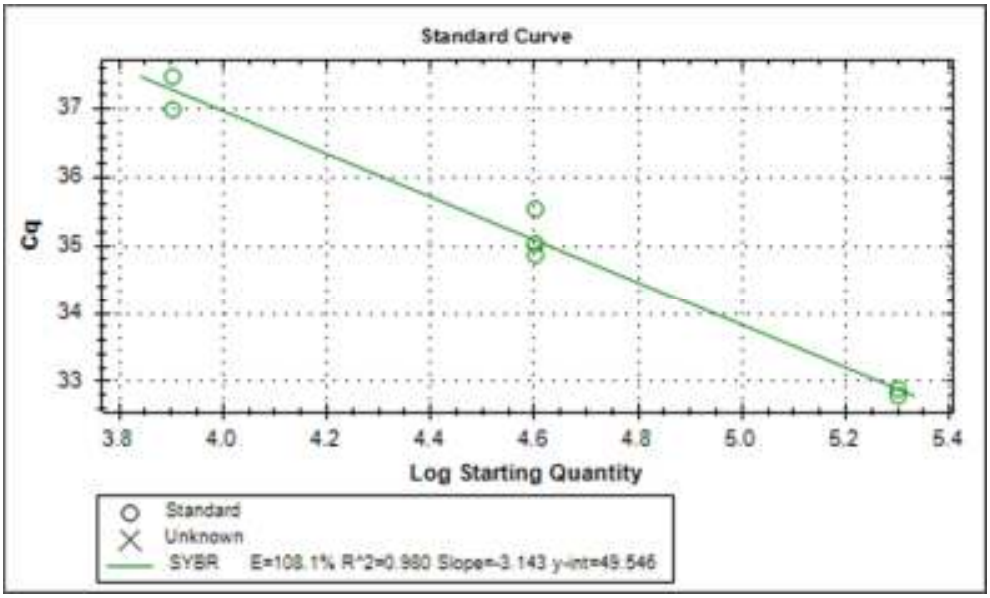

Figure A4. Standardization curve for Tbet.

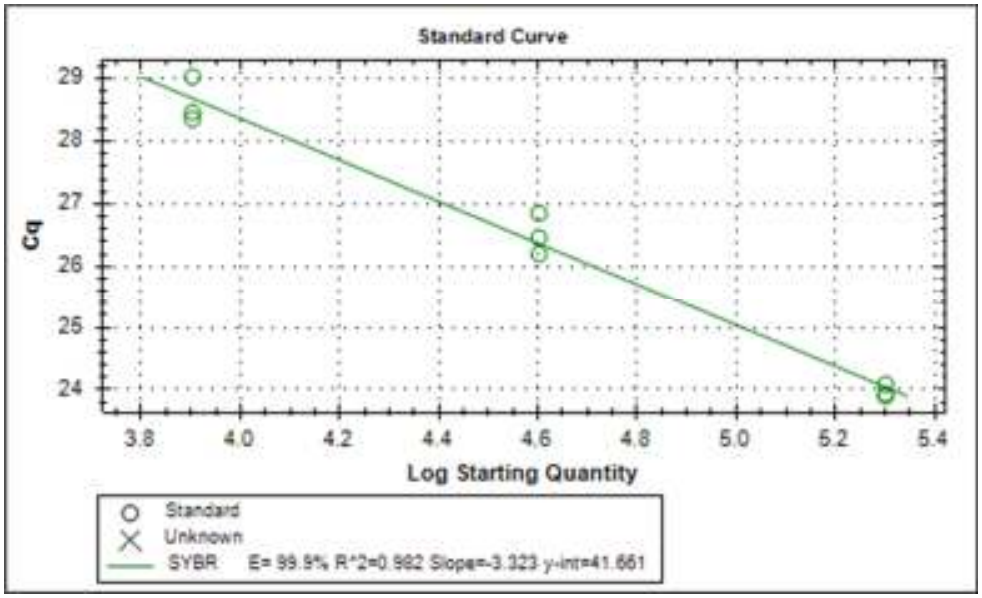

Figure A5. Standardization curve for STAT1.

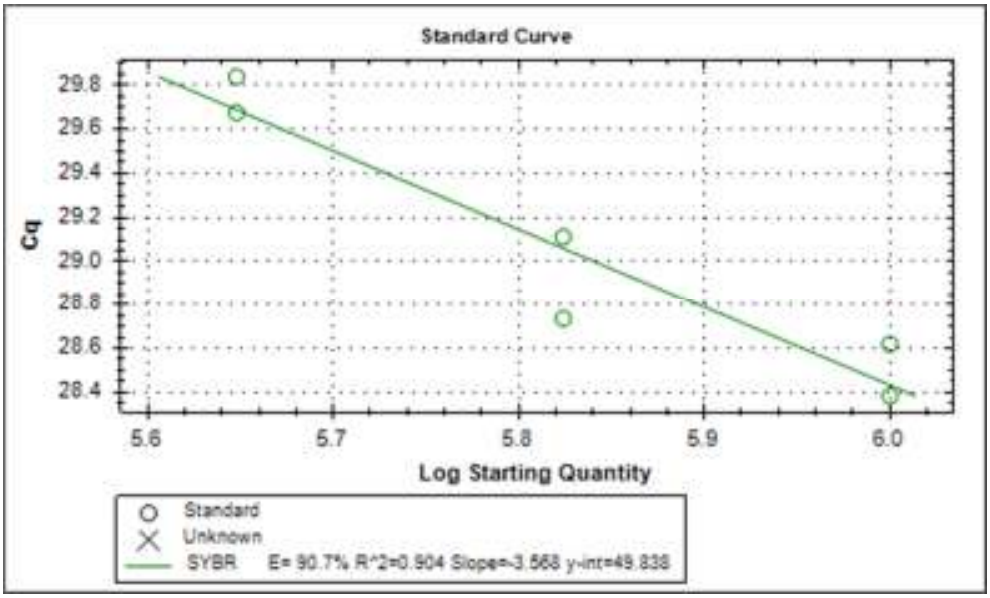

Figure A6. Standardization curve for STAT6.

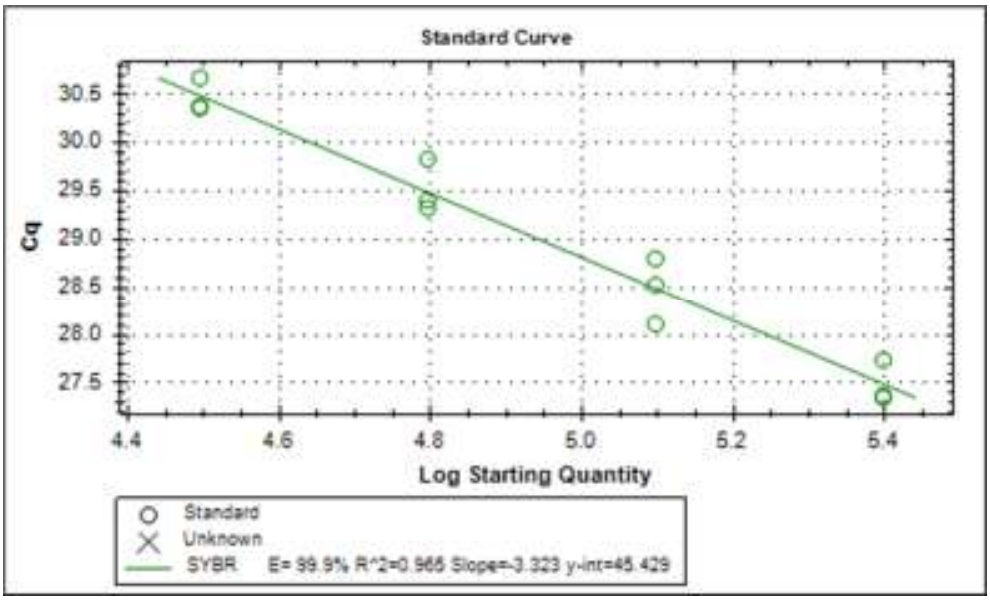

Figure A7. Standardization curve for GranzymeB.

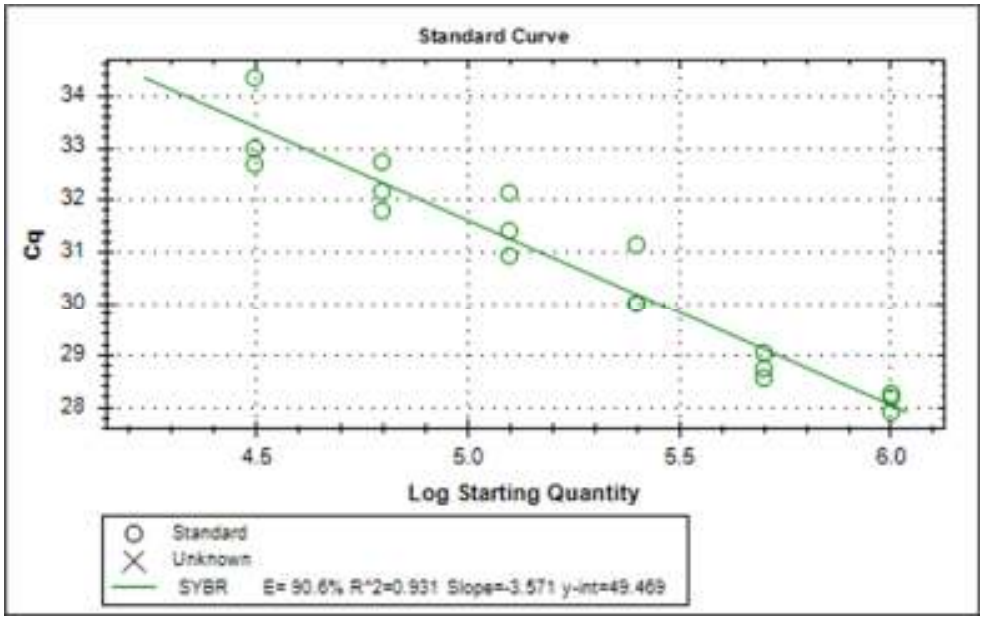

Figure A8. Standardization curve for Perforine.

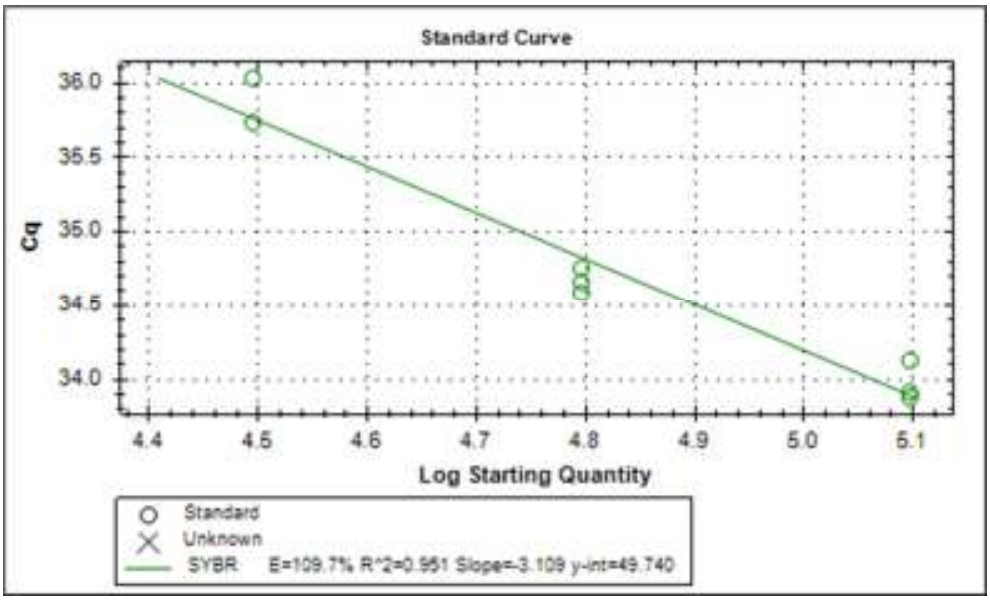

Figure A9. Standardization curve for KIR

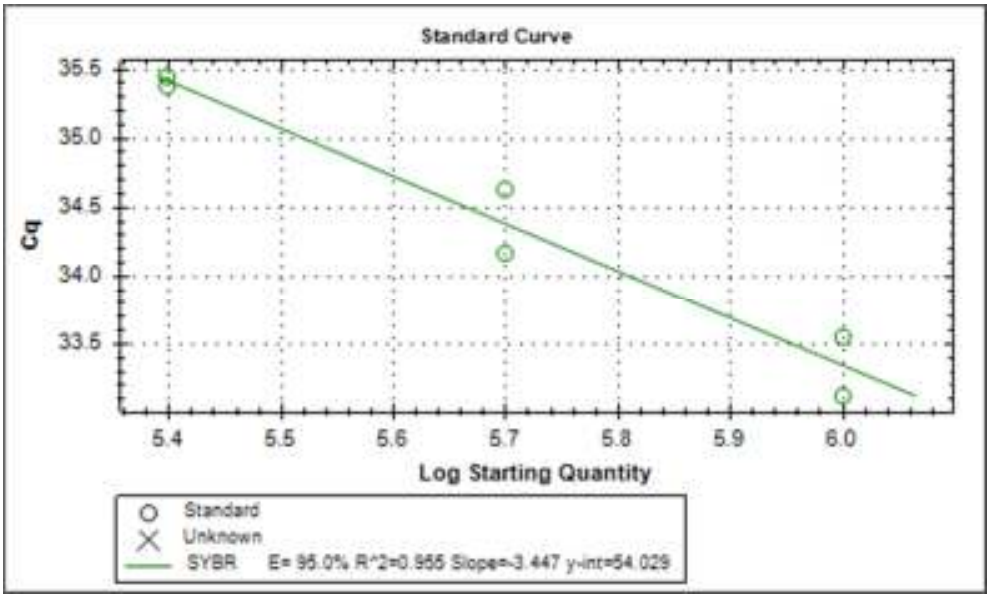

Figure A10. Standardization curve for HpRT.

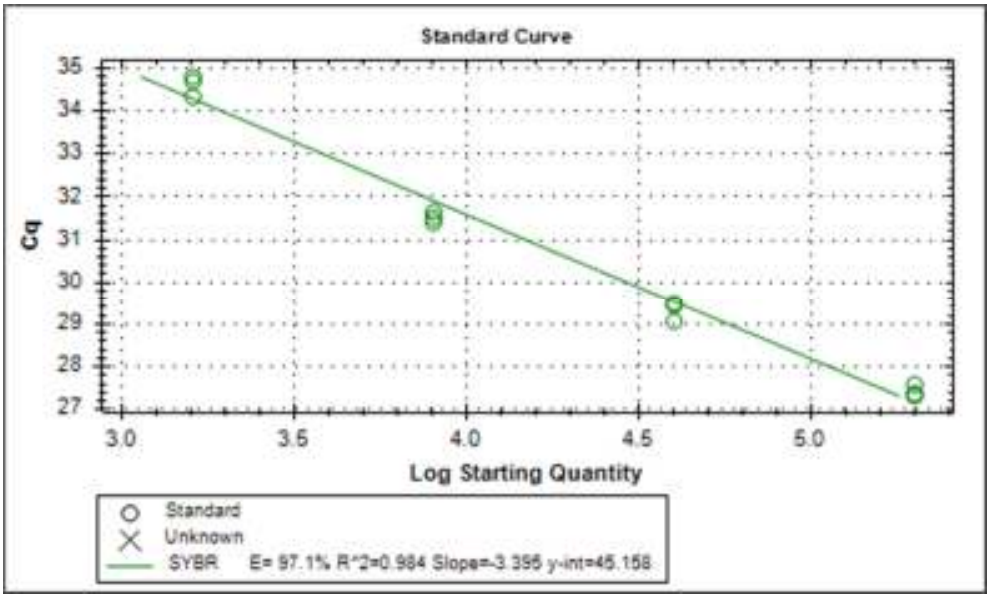

Figure A11. Standardization curve for RPS5.

Retrotranscription was performed in Corbett thermocycler (Corbett Life Sciences) using a RT kit (Qiagen) as per the manufacturer's instructions. Quantitative PCR consisted in one step at 95ºC for fifteen minutes, followed by 40 cycles corresponding to 15 seconds at 95º, one minute at 55ºc (in which, the plate was read) and step at 72ºC for 1 minute, a ending cycle at 95ºC for 15 seconds and a final step for the melting curve from 60 to 90ºC (0.5ºC of increase and 15 second of wave length measurement for each). Each reaction included cDNA (1:4 to 1:16 of 10 ng/ µL of retrotranscripted RNA), and SYBR®Green mix (Thermo Fisher).

ANNEX I