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ORIGINAL PAPER

Cytokine and acute phase protein expression in blood samples of harbour seal pups

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Abstract Cytokines and acute phase proteins (APP) are important mediators of the immune system and sensitive parameters to assess the immune status of mammals. Especially in newborn animals, maturation of the immune system is important for their survival. However, only little information is available for pinnipeds. To get baseline levels and investigate the development of the immune system, cytokine and APP expression was analysed in blood samples of six and eight harbour seal pups collected at the German North Sea Coast in 2004 and 2005, respectively. Blood samples were taken at admission and after rehabilitation in the Seal Centre Friedrichskoog. mRNA expressions of interleukin (IL)-1β, 2, 4, 6, 8, 10, 12, interferon (IFN)γ, and transforming growth factor (TGF)β, as well as of APP haptoglobin (HP), heat shock protein (HSP) 70 and metallothionein (MT) 2 were analyzed using real time RT-PCR. Cytokine and APP expression varied between animals and years. However, general changes during rehabilitation of pups were present. Higher levels of pro-inflammatory cytokines IL-1β, 6, 8, and 12 were found at admission, consistent with an activated immune system, whereas the anti-inflammatory cytokine IL-4 was increased after rehabilitation, suggesting recovery from infections and maturation of the immune system during rehabilitation. This preliminary study suggests that cytokines are sensitive parameters to assess the immune system of harbour seals.

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Introduction

Cytokines are important mediators of the immune system (Di Piro 1997; Elenkov and Chrousos 1999; Kidd 2003; Lucey et al. 1996; Mosmann and Sad 1996). Differentiation in pro-inflammatory cytokines, such as interleukin (IL)-1β, 6, 8, 12, and tumour necrosis factor (TNF)α, and anti-inflammatory cytokines, like IL-4 and 10, is reported (Elenkov and Chrousos 1999; Kidd 2003; Mosmann and Sad 1996). Furthermore, for medical purposes, cytokine expression is used to differentiate lymphocyte subpopulations. IL-2 and interferon (IFN)γ are marker for T helper cells-1 (Th-1), whereas IL-4, 5, 10, 13, and transforming growth factor (TGF)β are characteristic for T helper cells-2 (Th-2) (Elenkov and Chrousos 1999; Kidd 2003).

Pro-inflammatory cytokines, IL-1β, 6 and TNFα, stimulate acute phase protein (APP) expression, such as haptoglobin (HP), heat shock protein (HSP) 70 and metallothionein (MT) 2. Liver is the primary site of APP synthesis, but production in macrophages and lymphocytes has also been reported (Allan et al. 2000; Kostro et al. 2001). APP serve different physiological functions for the immune system and are used as marker for infection and

stress (Kostro et al. 2002; Murata et al. 2004; Petersen et al. 2004). HP inhibits production of pro-inflammatory, but not anti-inflammatory cytokines in human monocytes and affects respiratory burst activity of human neutrophils (Oh et al. 1990; Arredouani et al. 2005). MT 2 is important in acute phase response, detoxification of heavy metals and scavenging of free radicals (Bremner and Beattie 1990; Davis and Cousins 2000). The APP HSP70 is a group of conserved proteins and is involved in immune responses to several bacterial, viral and parasitic pathogens (reviewed by Young 1990; Njemini et al. 2002).

Cytokines and APP as important mediators of the immune system are used to assess the immune status in mammals. Several studies investigated cytokine-like activities or cytokine/APP expression in whales or dolphins (Beineke et al. 2004, 2006; Denis and Archambault 2001; Fonfara et al. 2007a, b; Funke et al. 2003; Inoue et al. 1999a, b, c, 2001; Ness et al. 1998; Shoji et al. 2001; St-Laurent et al. 1999). For harbour (*Phoca vitulina*) and grey seals (*Halichoerus grypus*), IL-1-like activity in leukocytes was reported (King et al. 1995). IL-2 was analysed in northern elephant seals (*Mirounga angustirostris;* Shoda et al. 1998), grey seals (St-Laurent et al. 1999) and harbour seals (Di Molfetto-Landon et al. 1995). IL-2 and 4 were used to specify metal-induced hypersensitivities in a grey seal (Kakuschke et al. 2006). IL-6-like activities and sequencing of IL-6 cDNA fragments were obtained for harbour and grey seals (King et al. 1993, 1996). Heavy metal-induced MT expression in peripheral blood leukocytes of grey seals was reported by Pillet et al. (2002). To the authors' knowledge, only three cytokines (IL-1, 2, and 6) are reported for harbour seals. Analysis of a wide range of cytokines and APPs is missing.

Furthermore, only few studies investigated newborn seals. Maturation of the immune system is crucial for harbour seal pup's survival. At birth, the immune system of marine mammals is not completely developed, reflected by low-immunoglobulin levels (King et al. 1998; Ross et al. 1993, 1994). As a temporary protection, pups receive maternal antibodies via colostrum (King et al. 1998; Ross et al. 1993, 1994). Incomplete development of the immune system in newborn grey seals was shown by reduced phagocytic activity of macrophages and reduced lymphocyte transformation (Lalancette et al. 2003). However, newborn harbour seals revealed strong mitogen-induced lymphocyte proliferation, consistent with efficient T-lymphocyte function (Ross et al. 1993, 1994; Kakuschke et al. 2008). These findings support a relative immunocompetence of harbour seal newborns as reflection of evolutionary adaptation to short nursing periods and limited maternal care (Ross et al. 1994).

In this study, quantitative RT-PCR for several cytokines and APP was established. Purpose of the study was to investigate the baseline expression of cytokines and APP in newborn harbour seal pups, the progression within the first months of life and to obtain possible markers of the immune status. To the authors' knowledge, this is the first report of GAPDH, IL-4, 8, 10, 12, IFNγ, TGFβ, MT and HSP70 amplification in harbour seal blood.

Materials and methods

Animals

Six and eight abandoned harbour seal pups were collected during birth period from June until July at the German North Sea coast line in 2004 and 2005, respectively. Pups, which were likely to survive, were rehabilitated in the Seal Centre Friedrichskoog. A veterinary examination of each seal pup, including weight, length, body condition, body temperature, physical examination and blood sampling, was performed immediately after admission. EDTA blood was taken from the epidural vertebral vein for haematology and mRNA isolation. A second blood sample of the same animals was taken after 2–3 months in the Seal Station just before their release back into the North Sea. At that time point the animals have gained a body weight of at least 25 kg and revealed no clinical signs of disease. Blood samples were processed within 1 hour after sampling. Haematology performed by Dr. Driver (Kleintierpraxis, Reinsbuettel, Germany) revealed white blood cell count of all animals within normal ranges (6–12 $\times$ 10^3 mm^{-3}; Siebert et al. 2006). Details of the animals investigated are listed in Table 1.

Primer design

Primers for the detection of IL-1β, 2, 4, 6, 8, 12, IFNγ and HP, HSP, MT2 were selected from conserved nucleotide sequences of published sequences. Sequences of different species (depending on availability bovine, ovine, canine, mice, equine) were compared choosing regions of great homology. Primer sequences, annealing temperatures, and length of amplicons are listed in Table 2. For the housekeeping gene GAPDH, for IL-10 and TGFβ, published primer pairs for harbour porpoises (*Phocoena phocoena*) from Beineke et al. (2004) were used.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from 300 µl EDTA blood by Ambion blood kit (Ambion Europe, Huntington, Cambridgeshire, UK). RNA isolation was performed according to the manufacturer's protocol. After DNase treatment (Ambion Europe, Huntington, Cambridgeshire, United

Table 1 Animal number, sex, dates (of admission and release; dd/mm/yy), age (at admission and release), white blood cell count (at admission and release; WBC), weight, length and body temperature of pups used for cytokine/acute phase protein detection

Animal	Sex	Admission						Release		
		Admission date	Age (days)	WBC ($\times 10^9$ L^{-1})[b]	Weight (kg)	Length (cm)	Body temperature (°C)	Release date	Age (days)	WBC (x10^9 L^{-1})[b]
Pv 1	F	21/06/04	7	9.7	8.9	58	37.0	26/08/04	73	10.2
Pv 2	F	21/06/04	7	8.8	8.6	57	37.2	14/09/04	92	12
Pv 3	F	21/06/04	7	6.5	8.9	57	36.6	26/08/04	73	6.3
Pv 4	F	21/06/04	7	8.6	10.5	59	37.4	14/09/04	92	9.1
Pv 5	M	21/06/04	10	8.1	10.9	65	37.0	26/08/04	76	7.5
Pv 6	M	21/06/04	7	9.7	8.7	54	36.4	26/08/04	73	9.8
Pv 7	F	07/06/05	3	4.7	7.2	54	36.6	04/09/05	92	9.9
Pv 8	F	07/06/05	3	9.9	9.5	60	36.8	25/08/05	82	7.2
Pv 9	F	16/06/05	7	8.8	8.7	53	38.2	25/08/05	77	11.3
Pv 10	F	22/06/05	5	7.2	10.1	49	38.8	25/08/05	69	7.5
Pv 11	F	23/06/05	7	6.8	8.4	52	37.8	18/09/05[a]	94	8.6
Pv 12	F	25/06/05	7	5.1	7.7	48	37.5	04/09/05	78	8.8
Pv 13	F	01/07/05	6–7	6.6	9.3	56	37.7	04/09/05	71–72	7.5
Pv 14	F	03/07/05	10–12	12.1	8.6	53	37.5	04/09/05	73–75	9.7

Pv Phoca vitulina, F female, *M* male, *WBC* white blood cells

[a] Pv 11 stayed in the Seal Station until 21/11/2005. Blood sample taken on 18/09/2005 was for routine re-investigation

[b] Normal range of WBC for harbor seals 6–12$\times 10^3$ mm^{-3} (Siebert et al. 2006)

Table 2 Primer sequences used for the amplification of the house keeping gene, cytokine and acute phase protein transcripts

Gen	Primer sequences (5′–3′)		Length of amplicon (bp)	Annealing temperature (°C)	Accession number
	s	as			
House-keeping gene					
GAPDH	GGG GCC ATC CAC AGT CTT CT	GCC AAA AGG GTC ATC ATC TC	185	57	AY919320
Cytokines					
IL-1β	GGC ATT TCG TGT CAG TCA TT	GCT CTT CAG GTC ATC CTC CT	90	52	AY919321
IL-2	CTT GCA TCG CAC TGA CTC TT	GCT CCA ACT GTT GCT GTG TT	89	55	AY919322
IL-4	CCT CCC AAC TGA TTC CAA CT	GAC AAA GGT GCT GGT GAG TG	74	50	AY919338
IL-6	TGG TGA TGG CTA CTG CTT TC	GGA GAG GTG AGT GGT GGT CT	61	54	AY919323
IL-8	ACA CAC TCC ACA CCT TTC CA	AGG CAC ACC TCA TTT CCA TT	149	50	AY919324
IL-10	CCT GGG TTG CCA AGC CCT GTC	ATG CGC TCT TCA CCT GCT CC	206	57	AY919326
IL-12	TTC TGG ACG TTT CAC ATG CT	CAC TCT GAC CCT CTC TGC TG	102	53	AY919327
IFNγ	GCA AGG CGA TAA ATG AAC TC	TGC GGC CTC GAA ACA GAT	98	50	DQ118388
TGFβ	TTC CTG CTC CTC ATG GCC AC	GCA GGA GCG CAC GAT CAT GT	392	58	AY919328
Acute phase proteins					
HP	CTG GCA GGC TAA GAT GGT TT	GTC AGC AGC CAT TGT TCA TT	75	54	AY919335[a]
HSP70	GGG GCT GAA CGT GCT GAG G	CCG CTT GTT CTG GCT GAT GTC	280	60	DQ118386
MT2	AAG GGC GCA TCG GAC AAG T	GAC CCA AAA CAA AAA GCA CCA A	91	55	AY919325

Base pair length, annealing temperature and GeneBank accession numbers for seal nucleotide sequences of this study are listed

GAPDH Glyceraldehyde-3-phosphate dehydrogenase, *IL* interleukin, *INF* interferon, *TGF* transforming growth factor, *HP* haptoglobin, *HSP70* heat-shock-protein 70, *MT* metallothionein, *s* sense, *as* antisense, *bp* base pair

[a] HP primer pairs published for harbor porpoises were used

Kingdom), 80–100 ng µl^{-1} RNA was reverse transcribed with murine reverse transcriptase (RT-PCR Core Kit, Applied Biosystems; Weiterstadt, Germany) and the resulting cDNA served as a template for real-time PCR using the thermocycler MX4000 (Stratagene Europe, Amsterdam, Netherlands). For real-time quantification, the

Brilliant SYBRGreen QPCR Master Mix (Stratagene Europe, Amsterdam, Netherlands) was used. It contained SYBRGreen I, as a fluorescence dye, dNTPs, $MgCl_2$ and a hot start Taq DNA polymerase. The fluorescence response was monitored in a linear fashion as the PCR product was generated over a range of PCR cycles. For each cytokine and APP, a dilution series from 10^9 copies to 10^2 copies was prepared, which served as a standard curve. The PCR started with an initial step at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 1 min, annealing temperature for 30 s, and elongation at 72°C for 1 min. The annealing temperatures are shown in Table 2. The fluorescence was measured at the end of the annealing and at the end of the dissociation program at a wavelength of 530 nm. To exclude measurement of non-specific PCR products and primer dimers and to determine a true amplification, the PCR was followed by a dissociation program for 1 min at 95°C, succeeded by 41 cycles during which the temperature was enhanced each cycle, starting at 55°C and ending at 95°C. Only PCR reactions with one well-defined peak were used for analysis. All PCR reactions were performed in duplicates. The constitutively expressed house-keeping gene GAPDH was used as control gene and to normalize cytokine and APP expression. Cytokine and APP expression index (EI) was calculated as follows:

$$\mathrm{EI} = \frac{\text{Number of cytokine copies}}{\text{Number of GAPDH copies}} \times 10^6$$

To verify the true amplification of cytokines and APPs, the PCR-products were sequenced (Seqlab Laboratories, Göttingen) and sequences were published in GenBank (NCBI, Table 2).

Results

Six and eight harbour seal pups were investigated and sampled in 2004 and 2005, respectively. GAPDH as house keeping gene was amplified in all blood samples. Interestingly, mRNA of each cytokine and APP investigated was present in all blood samples obtained. Sequencing of PCR products revealed the appropriate cytokine, which confirms the use of this method to detect cytokine/APP expression in seal blood samples.

Cytokine expression

In 2004, expression of IL-1β, 6, 8 and 12 mRNA decreased between admission and release in the majority of pups (Fig. 1). An exception was Pv 4, where an increase of IL-1β, 6, and 8 was found. This pup (Pv 4) revealed also higher mRNA amounts in comparison to other seals of this study. Additionally, APP mRNA expression increased over the time period of investigation (see also below).

For IL-2, 4 and IFNγ, an increase in mRNA expression was detected in most animals (Fig. 1). Exceptions were IFNγ in Pv 3, and IL-2 in Pv 2, where lower levels were found at release. IL-10 mRNA increased in two pups (Pv 2, 3), whereas four pups (Pv 1, 4, 5, 6) revealed a reduction comparing admission and release (Fig. 1).

The TGFβ mRNA expression decreased between admission and release in blood samples of three pups (Pv 1, 3, 5), and increased in three other pups (Pv 2, 4, 6) (Fig. 1).

In 2005, cytokine expression was highly variable between the animals. Comparing expression of IL-1β, 2, 4, 6, 8, and IFNγ at arrival and before release, a wide variation of cytokine levels were present at arrival, which was reduced at release (Fig. 3).

IL-1β, 6, 8 and 12 mRNA expression decreased in most pups between admission and release, similar to 2004 (Fig. 3). In detail, for IL-1β decreasing levels were found in four of eight pups (Pv 8, 9, 11, 12). IL-6 mRNA was reduced in five of eight pups (Pv 7, 8, 9, 11, 13), and IL-8 and 12 in four of eight pups (Pv 8, 9, 11, 13) at release.

IL-4 mRNA amounts increased in five of eight pups (Pv 9, 10, 11, 12, 14) (Fig. 3). IL-2 and IFNγ mRNA increased in the majority of pups (Fig. 3). In detail, six of eight pups (Pv 9, 10, 11, 12, 13, 14) revealed an increase in IL-2 mRNA, five of eight pups (Pv 9, 10, 11, 12, 14) showed an increase in IFNγ mRNA.

IL-10 mRNA levels were low in comparison to 2004 and increased from admission to release in blood samples of four pups (Pv 7, 9, 10, 14). Two pups (Pv 8, 11) showed a decrease and two pups only minor differences (Pv 12, 13) in IL-10 mRNA expression (Fig. 3). TGFβ mRNA expression decreased during rehabilitation in two pups (Pv 8, 11), and were increased or unchanged in six other pups (Pv 7, 9, 10, 12, 13, 14) (Fig. 3).

APP expression

In 2004, mRNA expression of APP HP and HSP increased between admission and release (except for Pv 1; Fig. 2). MT2 mRNA increased in three pups (Pv 2, 4, 6) and decreased in three other pups (Pv 1, 3, 5; Fig. 2). As mentioned before, Pv 4 showed an increase in all APP and higher HSP and MT2 levels than other pups at release (Fig. 2).

In 2005, higher HP mRNA levels were detected for several pups in comparison to 2004 (Fig. 4). Furthermore, expression of HP was highly variable between animals. HSP mRNA decreased during rehabilitation in majority of pups (Pv 8, 9, 11, 12, 13), which was in contrast to 2004, where an increase was detected. MT2 mRNA expression

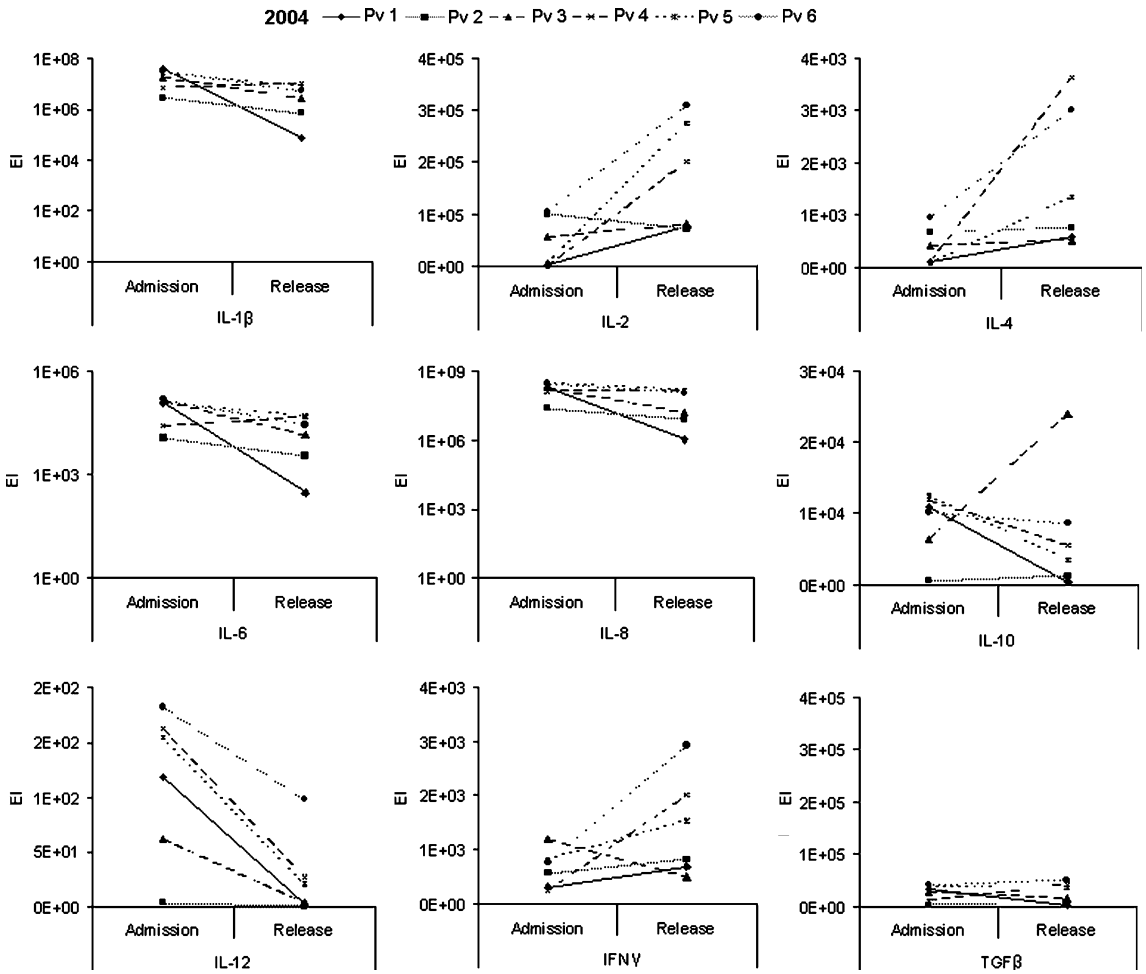

Fig. 1 Expression index (EI) of *IL-1β*, *2*, *4*, *6*, *8*, *10*, *12*, *IFNγ*, and *TGFβ* in blood samples of six harbour seal pups (*Pv 1–Pv 6*) taken at *admission* and before *release* in 2004

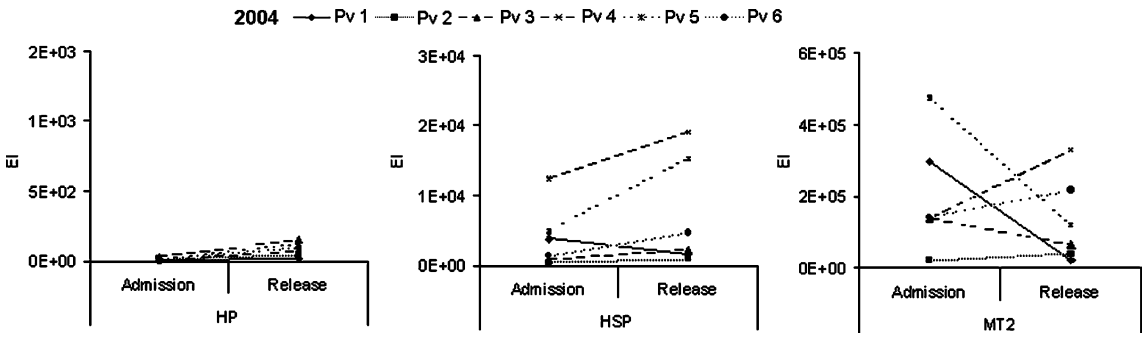

Fig. 2 Expression index (EI) of haptoglobin (HP), heat shock protein 70 (HSP) and metallothionein 2 (MT2) in blood samples of six harbour seal pups (*Pv 1–Pv 6*) taken at *admission* and before *release* in 2004

revealed less variability between the animals. Majority of animals showed lower MT2 mRNA amounts in comparison to animals investigated in 2004. However, a reduction of MT2 mRNA in most animals (Pv 7, 8, 9, 10, 11, 12) was obvious.

Discussion

This study showed successful amplification and sequencing of the house-keeping gene GAPDH, several cytokines and APP in harbour seal pups. This method was described

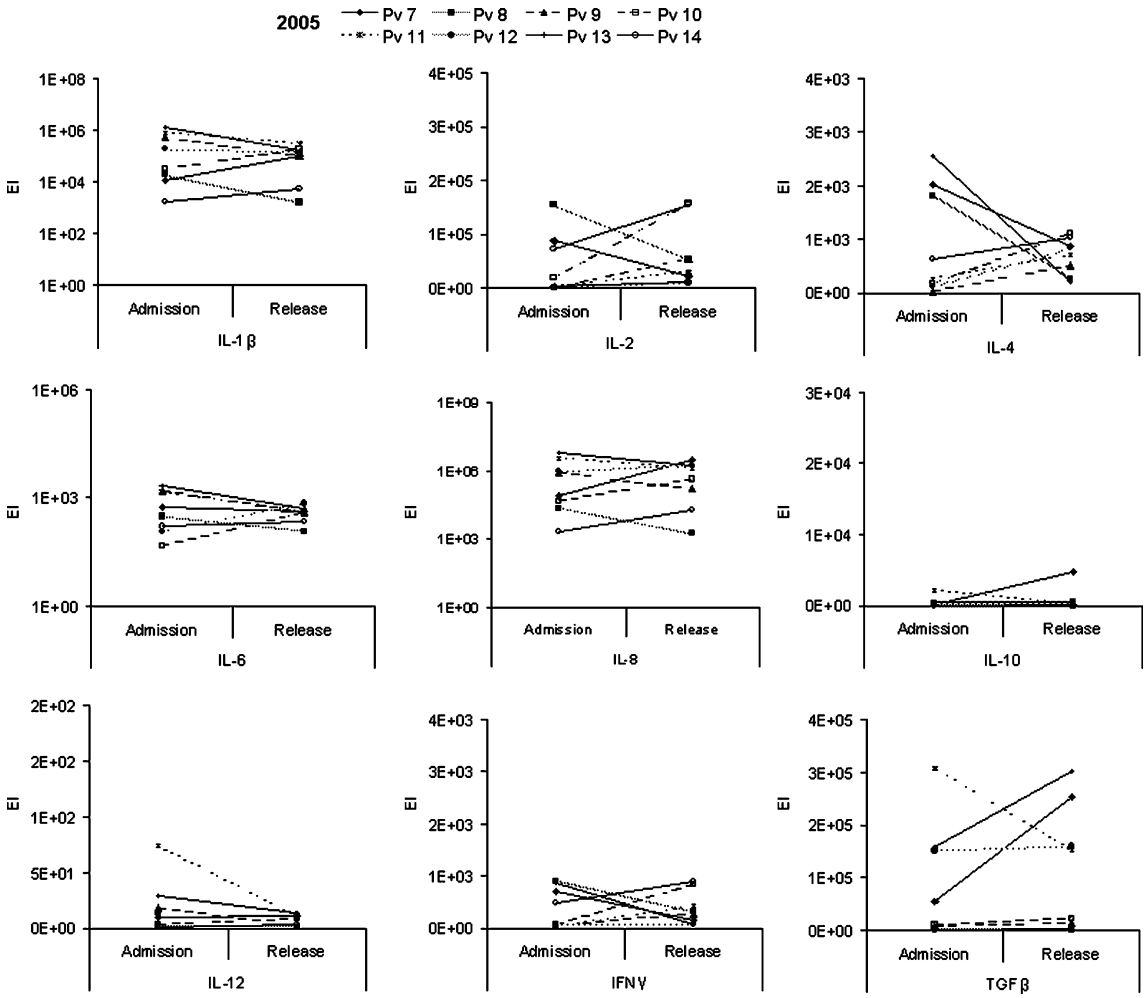

Fig. 3 Expression index (EI) of *IL-1β*, *2*, *4*, *6*, *8*, *10*, *12*, *IFNγ*, and *TGFβ* in blood samples of eight harbour seal pups (*Pv 7–Pv 14*) taken at *admission* and before *release* in 2005

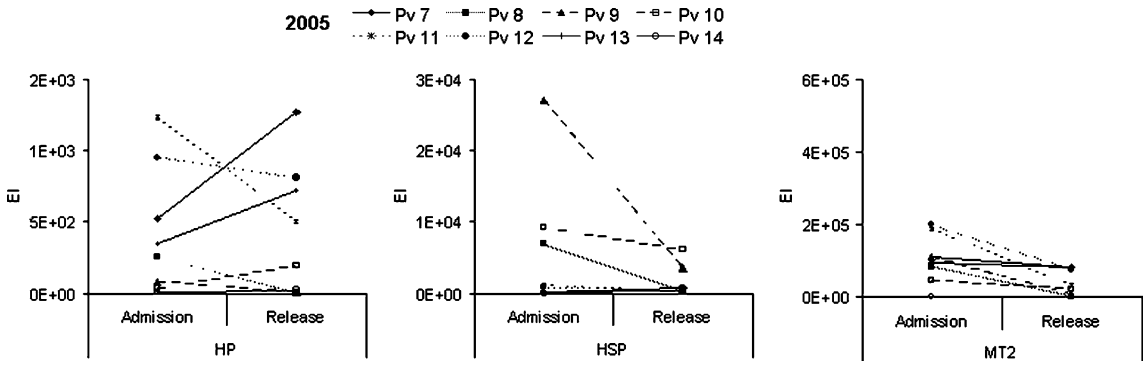

Fig. 4 Expression index (EI) of haptoglobin (HP), heat shock protein 70 (HSP) and metallothionein 2 (MT2) in blood samples of eight harbor seal pups (Pv 7–Pv 14) taken at *admission* and before *release* in 2005

before for other marine mammals (Beineke et al. 2004, 2006; Fonfara et al. 2007a, b; Kakuschke et al. 2006).

Maturation of the immune system is crucial for harbour seal pup survival. Cytokines and APP are important mediators and sensitive parameters of the immune system. To get baseline levels cytokine and APP expression and to improve the evaluation of pups abandoned by their mothers, several parameters were investigated. Analysing

cytokine and APP mRNA in newborn seals revealed individual variations between animals and years. Individual differences on cytokine expression and several impact factors are reported (Di Piro 1997; Elenkov and Chrousos 1999; Elenkov et al. 2005; Iwakabe et al. 1998; Kidd 2003; Lucey et al. 1996). However, general changes in cytokine and APP expression during rehabilitation of pups were obvious and single animals with different expression pattern were identified. Unfortunately, follow-up investigations of these pups to evaluate their progression after rehabilitation were not possible.

In 2004, cytokine mRNA expression pattern was similar for all seal pups. These animals were of similar weight and age and were collected at two consecutive days; suggesting similar environmental conditions (e.g. weather). Whereas in 2005, animals varied in age and weight and were collected and sampled over 1 month, which resulted in higher variability in cytokine and APP expression. This is consistent with the findings reported before (Di Piro 1997; Elenkov and Chrousos 1999; Elenkov et al. 2005; Iwakabe et al. 1998; Kidd 2003; Lucey et al. 1996).

However, most pups revealed higher levels of pro-inflammatory cytokines at admission in the Seal centre. These values decreased during rehabilitation, whereas anti-inflammatory cytokines, IL-4 and 10, increased during this time period. As baseline cytokine levels are not known, an increase or reduction into physiological levels or changes consistent with pathological conditions are possible. For harbour porpoises, elevated pro-inflammatory cytokines are found during suspected infection (Fonfara et al. 2007b). In people, bacterial infection and malnutrition resulted in increased IL-1, 6, 8, and CPR levels (Enwonwu et al. 2005; Yeung et al. 2004). Therefore, elevated IL-1, 6, 8, and 12 levels found in pups at admission might be consistent with poor body condition and infection. An infection was not confirmed by WBC. However, changes in cytokine expressions are likely to occur before changes in WBC are obvious (Di Piro 1997; Elenkov and Chrousos 1999; Elenkov et al. 2005; Kidd 2003).

IL-2, 4, and IFNγ mRNA expression increased during rehabilitation, in particular in animals sampled in 2004. Age dependent upregulation of IL-2, 4, and IFNγ, consistent with physiological maturation of the humoral and cellular immune response was reported by Härtel et al. (2005). Clinical implications or physiological variation was suspected for subjects who failed to produce IL-2, 4, or TNFα (Härtel et al. 2005). Similar results were detected in seal pups of this study. Recovery from infections and maturation of the immune system during rehabilitation is expected (Hall 1998; King et al. 1998; Lalancette et al. 2003; Marchant and Goldman 2005; Ross et al. 1993, 1994).

Different Th-cell subpopulations are described (Elenkov and Chrousos 1999; Kidd 2003). However, it was not possible to differentiate Th-cell subpopulations via cytokine expression in the seal pup blood samples. IL-2 and IFNγ as Th1 cytokines and IL-4 as Th2 cytokine were expressed at similar levels. Activation of both subpopulations or absence of Th subpopulations in seals is possible (Bremner and Beattie 1990; Kidd 2003; Mosmann and Sad 1996).

The APP mRNA expression was highly variable. In 2004, the expression of HP and HSP was associated with IL-2, 4 and IFNγ. HP as marker for infection and stress (Eckersall and Conner 1988; Heegaard et al. 1998; Murata et al. 2004; Petersen et al. 2004; Zenteno-Savin et al. 1997) and HSP, which is involved in immune responses to several bacterial, viral and parasitic pathogens (reviewed by Young, 1990; Njemini et al., 2002), might suggest progression of infection. However, this was not confirmed by other findings and the increase in HP might be caused by an increase into physiological levels, as HP mRNA levels in blood samples of 2004 were lower than that in 2005.

For most animals, in 2005 APP mRNA blood levels decreased during rehabilitation, which might be consistent with higher stress level or pathogen impact at admission and/or advanced immune responses at release (Cousins and Leinart 1988; Daffada and Young 1999; Heegaard et al. 1998; Murata et al. 2004; Petersen et al. 2004; Sato et al. 1994).

This preliminary study has several limitations. Interpretation of results is difficult as baseline cytokine and APP levels are not known. Follow-up investigations of animals were not possible and a control group did not exist. Only limited information of animals were available, the sample number was small and animals were sampled at different time points. Therefore, a differentiation of infection and/or stress on cytokine/APP expression or physiological maturation of the immune system was not possible. Further investigations of different groups (to investigate the influence of age, disease and environment) over a longer time period and systematic sampling are necessary.

This study revealed that cytokine and APP expression in seal pups varied between animals and years. Differences in health status, maturation of the immune system and ability to handle stress between animals were likely. However, our results suggest an activated immune system of harbour seal pups at admission to the Seal Station and recovery from infection and mature immune system after rehabilitation. These preliminary results suggest pro-inflammatory cytokines IL-1β, 6, 8, and 12 as well as the anti-inflammatory cytokine IL-4 as useful parameters to assess the immune system of harbour seals.

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