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journal or publication title	Cytokine
volume	65
number	1
page range	74-78
year	2014-01-01
URL	<a href="http://hdl.handle.net/2297/36274">http://hdl.handle.net/2297/36274</a>

doi: 10.1016/j.cyto.2013.09.007

## **Sustained elevation of serum interleukin-18 and its association with hemophagocytic lymphohistiocytosis in XIAP deficiency**

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**Running title:** Elevated IL-18 in XIAP deficiency

## **Abstract**

X-linked lymphoproliferative syndrome (XLP) is a rare primary immunodeficiency characterized by increased vulnerability to Epstein-Barr virus infection. XLP type 1 is caused by mutations in *SH2D1A*, whereas X-linked inhibitor of apoptosis (XIAP) encoded by *XIAP/BIRC4* is mutated in XLP type 2. In XIAP deficiency, hemophagocytic lymphohistiocytosis (HLH) occurs more frequently and recurrence is common. However, the underlying mechanisms remain mostly unknown. We describe the characteristics of the cytokine profiles of serum samples from 10 XIAP-deficient patients. The concentration of interleukin (IL)-18 was strikingly elevated in the patients presented with HLH, and remained high after the recovery from HLH although levels of other pro-inflammatory cytokines approached the normal range. Longitudinal examination of two patients demonstrated marked exacerbation of IL-18 levels during every occasion of HLH. These findings may suggest the association between HLH susceptibility and high serum IL-18 levels in XIAP deficiency.

## **Keywords**

X-linked inhibitor of apoptosis (XIAP); X-linked lymphoproliferative syndrome (XLP); interleukin-18; hemophagocytic lymphohistiocytosis (HLH)

## **Abbreviations**

XLP, X-linked lymphoproliferative syndrome; SLAM, signaling lymphocyte activation molecule; SAP, SLAM-associated protein; XIAP, X-linked inhibitor of apoptosis; HLH, hemophagocytic lymphohistiocytosis; FHL, familial hemophagocytic lymphohistiocytosis; EBV, Epstein-Barr virus; sJIA, systemic juvenile idiopathic arthritis; AOSD, adult-onset Still's disease; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; ATP, adenosine triphosphate; NOD2, nucleotide-binding and oligomerization domain 2 ; NK, natural killer.

## 1. Introduction

X-linked lymphoproliferative syndrome (XLP) is a rare primary immunodeficiency that is characterized by an extreme susceptibility to Epstein-Barr virus infection resulting in fatal infectious mononucleosis or hemophagocytic lymphohistiocytosis (HLH), hypogammaglobulinemia, and malignant lymphoma [1-3]. Most cases are caused by mutations in the *SH2D1A* gene, which encodes the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), a cytoplasmic adaptor molecule involved in intracellular signaling downstream of the SLAM family of surface receptors (XLP type 1). SAP is expressed in T, natural killer (NK), and invariant NKT cells [1-3]. A deficiency of X-linked inhibitor of apoptosis (XIAP) caused by *XIAP/BIRC4* gene mutations has been also identified to cause XLP (XLP type 2) [4]. XIAP, a member of the inhibitor of apoptosis family of proteins, plays an antiapoptotic role as a potent inhibitor of caspases 3, 7, and 9, and also possesses E3 ubiquitin ligase function [1-3]. In contrast to SAP, XIAP is expressed ubiquitously. Patients with XIAP deficiency have been reported to develop HLH more frequently compared with SAP deficiency [5]. However, it remains unknown why a deficiency of XIAP leads to HLH and its recurrence in patients with XIAP deficiency.

HLH is a heterogeneous group of diseases that are characterized by uncontrolled proliferation of activated macrophages and T cells with overproduction of pro-inflammatory cytokines [6, 7]. Patients with HLH may present with fever, cytopenia, hepatosplenomegaly, liver dysfunction, coagulation abnormalities, and hemophagocytosis. [6, 7] HLH is comprised of primary and secondary forms. Primary HLH includes familial

HLH (FHL), which is caused by genetic defects related to granule-dependent cytotoxicity, and immunodeficiencies, such as XLP. FHL type 2 (FHL2) due to perforin deficiency is the most common form of primary HLH [6, 7]. Perforin is a crucial effector molecule for cytotoxicity that is present in the granules of cytotoxic T cells and NK cells. Secondary HLH is associated with a variety of infections, autoimmune diseases and malignancies. Epstein-Barr virus (EBV)-associated HLH (EBV-HLH) is the most frequent subtype of secondary HLH in Japan [8]. Studies of cytokines from HLH patients have demonstrated elevated concentration of many pro-inflammatory cytokines, such as interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, and IL-18 [9-11].

IL-18 is a potent pro-inflammatory cytokine that was originally identified as an IFN- $\gamma$ -inducing factor [12, 13]. It has been reported that IL-18 induces either Th1 or Th2 polarization depending on the immunologic context, and promotes a variety of innate immune processes associated with infection, inflammation, and autoimmunity [12, 13]. In this report, we describe the characteristic cytokine profiles of XIAP deficiency, especially IL-18, which might be associated with its HLH susceptibility.

## **2. Methods**

### ***2.1. Patients***

We studied 10 patients with XIAP deficiency from 9 families (Table 1). Case reports of Pt2.1, Pt2.2, Pt4, Pt5, Pt8, and Pt9 have been described elsewhere [5, 14, 15]. Patients Pt2.1, Pt2.2, Pt4, and Pt5 were reported in a previous report as P6.1, P6.2, P1, and P3.1, respectively [14]. Patient Pt7 was a younger brother of P2.1 and P2.2 [14]. Patients Pt8 and Pt9 were reported in a previous report as Patient 3 and Patient 9, respectively [5]. All patients showed typical features of HLH, such as persistent fever, cytopenia, liver dysfunction, and hyperferritinemia during the acute phase of HLH. The age of onset was variable, ranging from early infancy to 8 years. Most patients were treated with corticosteroids with or without cyclosporine A, and showed recurrent HLH. Patient Pt3 underwent cord blood transplantation and is alive with no evidence of disease. Patients Pt8 and Pt9 underwent allogeneic bone marrow transplantation but died of complications [16]. Blood samples were collected during the acute phase of HLH and/or during a clinically stable phase. We also investigated 4 cases of SAP deficiency, 6 cases of FHL2, and 11 cases of EBV-HLH as disease controls [17]. No detectable mutations within the *SH2D1A* or *XIAP/BIRC4* genes were observed in the 5 male patients with EBV-HLH [18]. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

### ***2.2. Analysis of XIAP mutations and protein expression***

DNA was extracted from blood samples using standard methods. The *XIAP/BIRC4* gene was amplified from genomic DNA using specific primers [5, 14]. Sequencing was performed on purified polymerase chain reaction products using the ABI Prism BigDye Terminator Cycle sequencing kit on an ABI 310 or 3130 automated sequencer (Applied Biosystems, Foster, CA). Flow cytometric and Western blot analysis of intracellular XIAP were performed, as described previously [5, 14].

### ***2.3. Cytokine determination***

Serum concentrations of cytokines were determined using the following enzyme-linked immunosorbent assay kits: neopterin (IBL, Hamburg, Germany); IFN- $\gamma$ , IL-6, and TNF- $\alpha$  (R&D systems, Minneapolis, MN); and IL-18 (MBL, Nagoya, Japan) [17, 19]. Analysis of differences among groups was performed using the Student's *t*-test for unpaired samples. Differences with *p*-values less than 0.05 were considered significant.

### ***2.4. Cell cultures***

Peripheral blood mononuclear cells (PBMCs) were isolated from patients and controls by Ficoll-Hypaque gradient centrifugation. To stimulate monocytes, PBMCs were incubated with 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) with or without 5 mM adenosine triphosphate (ATP; Sigma-Aldrich) in RPMI 1640 medium containing 10% fetal calf serum and antibiotics [20]. After 24 hours, culture supernatants were collected and stored at -80°C until cytokine assay.



### **3. Results**

#### ***3.1. XIAP/BIRC4 mutations and protein expression***

As shown in Table 1, most patients had nonsense or frameshift mutations in the *XIAP/BIRC4* gene. Patients Pt1 and Pt3 were found to carry novel nonsense mutations, c.847C>T (Q283X) and c.664C>T (R222X), respectively. Intracellular XIAP was not detected in PBMCs from patient Pt1, whereas residual expression was demonstrated in patient Pt3. Patient Pt6 had a novel splice site mutation, c.1056+1G>A, resulting in no detectable expression of XIAP.

#### ***3.2. Cytokine profiles***

The concentration of IL-18 was markedly elevated in XIAP-deficient patients who presented with HLH (mean, 86500 pg/mL; Figure 1A). Children who were affected with SAP deficiency, FHL2 or EBV-HLH exhibited much less elevation of IL-18 with mean concentrations of 6600 pg/mL (range, 6200 - 7000 pg/mL), 3680 pg/mL (range, 1300 - 10500 pg/mL) and 4256 pg/mL (range, 3050 - 6950 pg/mL), respectively (Figure 1A) [17]. Other pro-inflammatory cytokines such as IL-6, neopterin, IFN- $\gamma$ , and TNF- $\alpha$  were elevated in patients with XIAP deficiency, however, levels of these cytokines were comparable to those of patients with SAP deficiency, FHL2, or EBV-HLH (Figure 1A). The levels of soluble IL-2 receptor were also elevated in patients with XIAP deficiency (mean, 2910 IU/mL).

As expected, after the recovery from HLH, the concentration of each pro-inflammatory cytokine declined in patients with SAP deficiency, FHL2 and EBV-HLH.

However, as XIAP-deficient patients recovered from HLH, serum IL-6, neopterin, IFN- $\gamma$ , and TNF- $\alpha$  levels approached the normal range but the levels of IL-18 remained high in these patients (mean, 4090 pg/mL; Figure 1A). Longitudinal examination of the cytokine profiles in patients Pt1 and Pt2.1 clearly demonstrated sustained elevation of IL-18 and its marked exacerbation on every occasion of recurrent HLH (Figure 1B).

### ***3.3. Secretion of IL-18 from PBMCs after in vitro stimulation***

To assess whether hypersecretion of IL-18 was observed from monocytes after inflammasome stimulation, PBMCs from normal individuals and 3 patients with XIAP deficiency (Pt1, Pt2.1, and Pt2.2) were cultured for 24 hours with LPS and ATP. Consistent with previous observations [20], IL-18 was efficiently secreted in normal PBMCs after stimulation with LPS plus ATP (Figure 2), although TNF- $\alpha$  and IL-1 $\beta$  were produced in response to stimulation with only LPS (data not shown). There was little production of IL-18 in PBMCs from patients with XIAP deficiency after stimulation with LPS as well as no stimulation, suggesting no spontaneous production of IL-18. More importantly, levels of IL-18 secretion after stimulation with LPS plus ATP in PBMCs from the patients were comparable to those of normal controls (Figure 2).

#### 4. Discussion

XIAP deficiency is characterized by a high incidence of HLH [1-3]. Most patients suffer from recurrent HLH, although the reason for the increase in HLH susceptibility remains unknown. To further characterize the disease, we investigated the cytokine profiles during the acute phase of HLH and during a clinically stable phase from 10 patients with XIAP deficiency and compared them to patients with SAP deficiency, FHL2 (perforin deficiency), and EBV-HLH.

Hypersecretion of pro-inflammatory cytokines from activated T cells and macrophages has been considered to account for the severe systemic symptoms of HLH [6, 7]. Indeed, during the acute phase of HLH, patients exhibited hypercytokinemia, i.e., IL-6, IL-18, neopterin, IFN- $\gamma$ , and TNF- $\alpha$ , regardless of underlying genetic disease. However, compared with our previous studies of cytokines in other forms of HLH [17], the concentration of IL-18 was strikingly elevated during HLH and remained high after the recovery from HLH in XIAP-deficient patients. Patients Pt2.2 and Pt5, both of whom had disease for longer than 10 years, also showed high IL-18 levels during the clinically stable phase, indicating that this characteristic appears to be consistent and stable in the patients. No correlation was observed between the concentration of IL-18 and the levels of residual XIAP protein expression. In addition, the level of IL-18 declined to within the normal range (142 pg/mL) in patient Pt3 only after successful stem cell transplantation. Taken together, these findings may indicate the association between HLH susceptibility in XIAP deficiency and high serum levels of IL-18 and the possible role of serum IL-18 as a marker of disease activity.

Similar cytokine profiles have recently been described in patients with systemic juvenile idiopathic arthritis (sJIA) and with adult-onset Still's disease (AOSD), which also showed persistent high levels of serum IL-18 [19, 21-26]. sJIA is a systemic inflammatory disease classified within the spectrum of JIA and is characterized by fever, rash and arthritis [12]. AOSD is likely to be the adult counterpart of sJIA [12]. In both diseases, macrophage activation syndrome, a form of HLH associated with rheumatic diseases, occurs frequently [12]. The concentration of IL-18 was further elevated in patients with sJIA/AOSD who presented with macrophage activation syndrome [19]. These profiles are quite similar to those of XIAP deficiency. Although the etiology of sJIA/AOSD remains unknown, these disorders might share a common final pathway of abnormal IL-18 secretion. On the other hand, studies of NK cells from patients with sJIA have demonstrated that a defect in IL-18 receptor  $\beta$  phosphorylation was involved in impaired NK cell function in sJIA [27]. Further studies are necessary to assess whether similar defects could exist and contribute to the disease pathogenesis in patients with XIAP deficiency. Moreover, it is tempting to speculate that XIAP-deficient patients might be misdiagnosed with sJIA/AOSD. In fact, our patient Pt1 was initially given the diagnosis of sJIA despite the absence of arthritis (case 4 in reference no. 7). Although arthritis may not be prominent, particularly early in the course of sJIA [28], patient Pt1 has shown no clinical or laboratory evidence of arthritis to date.

It is well known that IL-18 is produced mainly by monocytes/macrophages in response to a variety of stimuli, and that IL-18 is synthesized as a precursor molecule, which is cleaved by the caspase-1 within the inflammasome [12, 13]. However, the mechanism underlying the sustained elevation of IL-18 in XIAP deficiency is unclear at

present. The cellular source of the IL-18 also remains to be elucidated. Our preliminary experiments failed to demonstrate that PBMCs from XIAP-deficient patients secreted larger amounts of IL-18 upon stimulation with LPS and ATP, compared with normal controls. However, recent evidence suggests that XIAP mediates signaling of nucleotide-binding and oligomerization domain 2 (NOD2) in inflammation and innate immunity [29-31]. Because NOD2 can activate caspase-1, it is possible that XIAP is involved in inflammasome-mediated IL-18 production.

It is unknown why a deficiency of XIAP leads to the development of HLH [1-3]. Deficiency of XIAP has been observed to result in increased sensitivity of T cells to undergo reactivation-induced cell death [4, 5]. Therefore XIAP may function to prevent HLH by inhibiting the apoptosis of cells that mediate the clearance of pathogens. Decreased numbers of NKT cells may also contribute to the development of HLH similar to SAP deficiency (XLP type 1), although there is some controversy over the numbers of such populations [32]. In addition, the loss of XIAP may influence immune cell activation resulting in alterations in pro-inflammatory cytokine production and cell survival in murine studies [33-35]. Because hypersecretion of IL-18 has been reported to play important roles in the pathogenesis of HLH [36], sustained elevation of IL-18 and its marked exacerbation on HLH in patients with XIAP deficiency would be an additional possibility to explain HLH susceptibility in this disease.

In summary, our studies demonstrate markedly elevated serum levels of IL-18 that are associated with HLH in XIAP deficiency. Characterization of the mechanism underlying the hypersecretion of IL-18 could provide important insights into the pathogenesis of the disease.

## **Acknowledgments**

We thank Ms Harumi Matsukawa and Ms. Shizu Kouraba for their excellent technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant from the Ministry of Health, Labour, and Welfare of Japan, Tokyo.

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**Table 1. Patient characteristics.**

	Pt1	Pt2.1 <sup>b</sup>	Pt2.2 <sup>b</sup>	Pt3	Pt4	Pt5	Pt6	Pt7	Pt8	Pt9
Nucleotide mutation	c.847C>T	c.1021_ 1022delAA	c.1021_ 1022delAA	c.664C>T	c.712C>T	c.650delG	c.1056+1G>A	c.1141C>T	c.1445C>G	c.310C>T
Effect	Q283X	N341fs	N341fs	R222X	R238X	W217fs	splice defect	R381X	P482R	Q104X
Protein expression <sup>a</sup>	negative	residual	residual	residual	residual	negative	negative	negative	residual	negative
Age at onset	1 mo	17 mo	15 mo	5 mo	20 mo	2 mo	3 yr	8 mo	infancy	8 yr
Current age	5 yr	3 yr	15 yr	2 yr	7 yr	12 yr	9 yr	10 mo	deceased	deceased
Family history	No	Yes	Yes	No	No	Yes	No	Yes	No	Yes
HLH	+	+	+	+	+	+	+	+	+	+
Recurrent HLH	+	+	+	+	+	+	+	-	NA	+
Fever	+	+	+	+	+	+	+	+	+	+
Splenomegaly	-	+	+	+	+	-	-	+	+	+
Cytopenia	+	+	+	-	+	+	-	+	+	+
EBV	-	+	+	-	+	+	-	-	-	-
Hypogammaglobulinemia	-	-	-	-	-	-	-	-	-	+
Colitis	-	-	-	-	+	-	-	-	NA	NA

Lymphoma	-	-	-	-	-	-	-	-	-	-
Treatment	PSL, CsA	IVIG, Dex	PSL	Dex, CsA	PSL, CsA	PSL, CsA	PSL	Dex, CsA	HLH 2004, including IT treatment	Dex rituximab
Allogeneic HSCT	-	-	-	+	-	-	-	-	+	+

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<sup>a</sup>Intracellular expression of XIAP in lymphocytes was analyzed by flow cytometry or Western blot. <sup>b</sup> sibling cases.

HLH, hemophagocytic lymphohistiocytosis; mo, month; yr, year; NA, not applicable; EBV, Epstein-Barr virus; PSL, prednisolone; CsA, cyclosporin A; Dex, dexamethasone, IVIG, intravenous immunoglobulin; HSCT, hematopoietic stem cell transplantation.

## Figure Legends

### Figure 1. Cytokine profiles.

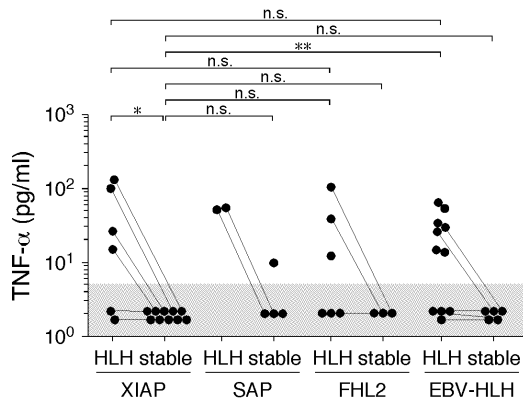
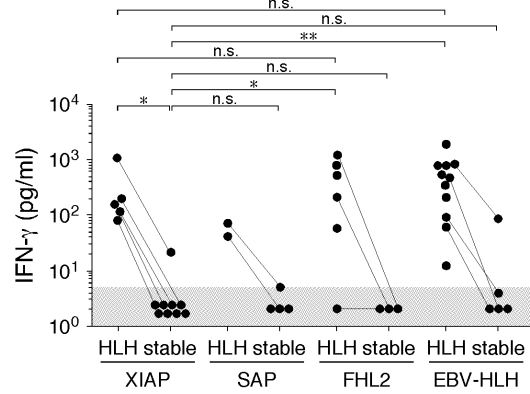
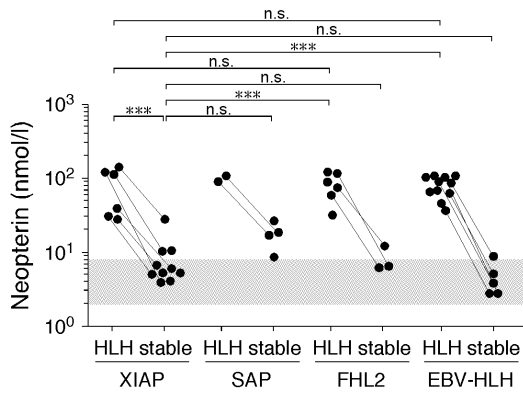
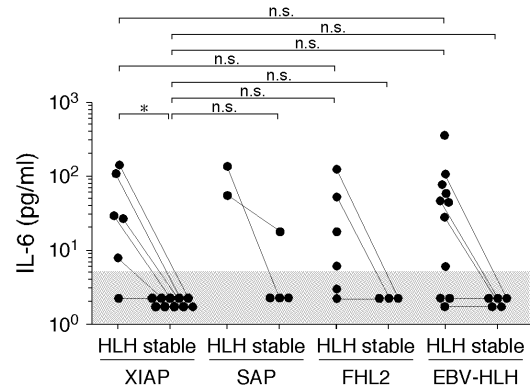
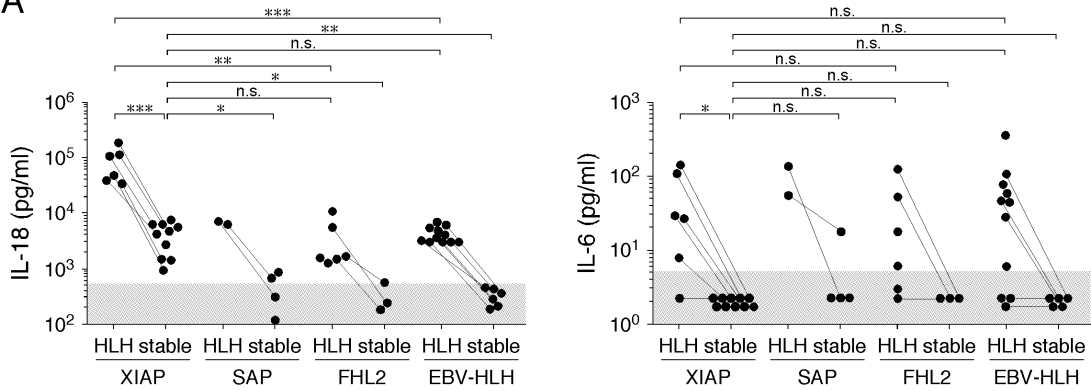
(A) Serum concentrations of interleukin (IL)-18, IL-6 and neopterin were measured in patients with X-linked inhibitor of apoptosis (XIAP) deficiency, with signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) deficiency, with familial hemophagocytic lymphohistiocytosis type 2 (FHL2) due to perforin deficiency, and with Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH). Shaded areas represent the ranges of the normal values. (B) Longitudinal examination of IL-18 levels. Arrows indicate episodes of HLH. HLH, hemophagocytic lymphohistiocytosis; n.s., not significant. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

### Figure 2. IL-18 secretion after in vitro stimulation.

Peripheral blood mononuclear cells were cultured with lipopolysaccharide (LPS) and adenosine triphosphate (ATP) for 24 hours. The levels of IL-18 in culture supernatants were measured by enzyme-linked immunosorbent assay.

Figure 1

A



B

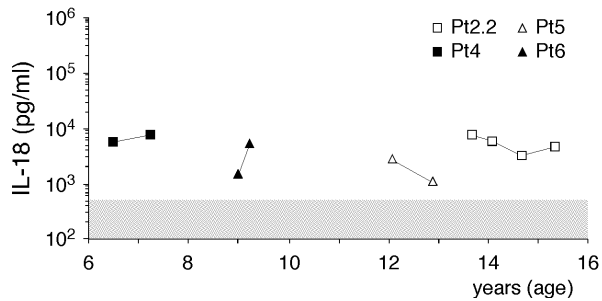
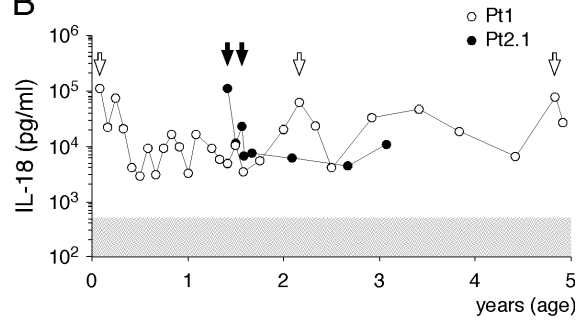




Figure 2

