Yusho Patients Show Increased Serum IL-17, IL-23, IL-1β, and TNFα Levels More Than 40 Years After Accidental Polychlorinated Biphenyl Poisoning

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Running title: IL-17, IL-23, IL-1β and TNFα levels in Yusho patients

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Abstract

The Yusho poisoning incident, caused by rice oil contaminated with polychlorinated biphenyls (PCBs), polychlorinated quarterphenyls (PCQs), and polychlorinated dibenzofurans (PCDFs) generated by heat-denatured PCBs, occurred in 1968 in western Japan. Although severe symptoms are rarely observed today, the levels of PCBs and PCDFs in the sera of Yusho patients remain high. The aryl hydrocarbon receptor (AhR), which also acts as a dioxin receptor, is a transcriptional regulator that mediates dioxin toxicity. Recent studies show that dioxin mediates its immune toxic effects via AhR and that AhR activation induces dysregulation of interleukin (IL)-17- producing T (T_H17) cells. We therefore hypothesized that Yusho patients would show dysregulated T_H17 cell-mediated immune responses. To validate the hypothesis, levels of IL-17 and IL-22, each secreted by T_H17 cells, along with IL-1β and IL-23 were measured in serum samples from 40 Yusho patients and 40 age-matched controls. Levels of tumor necrosis factor TNF)-α potentially secreted by T_H17 cell-stimulated neutrophils and macrophages were also measured. The results indicated that serum IL-17 levels, as well as those of IL-1β, IL-23, and TNFα, were significantly higher in Yusho patients than in controls. In contrast, serum IL-22 levels were significantly lower in the Yusho patients. These results suggest that Yusho patients have dysregulated T_H17 cell-mediated immune responses that may be linked to inflammation.

Introduction

The Yusho incident that occurred in western Japan in 1968 involved a mass poisoning of 2,000 individuals through contaminated food (Furue, 2005). This poisoning resulted from the accidental ingestion of rice bran oil contaminated with dioxin-like components, including polychlorinated biphenyls (PCBs). It was later found that the oil was also contaminated with polychlorinated dibenzofurans (PCDFs), quarterphenyls (PCQs), and other related compounds. Skin symptomae experienced by "Yusho patients" were acneiform eruptions and pigmentation of the skin, nails, and conjunctiva. Other symptoms included eye discharge, hyperemia, and eyelid swelling. In addition, general malaise, headache, nausea, vomiting, numbness of limbs, arthralgia, irregular menstruation, and growth retardation in infants and children were noticed. Most PCBs and PCDFs agents have a high affinity for fats. As such, they are not easily eliminated from the body and can be stored in adipose tissue for a long time (Furue, 2005). Therefore, even though more than 40 years have passed since the accidental poisoning, high PCBs, PCDFs, and PCQs levels are still detected in Yusho patients' sera. Furthermore, a number of these patients still suffer from many of the Yusho-specific symptoms mentioned above.

The aryl hydrocarbon receptor (AhR) that acts as a dioxin receptor also acts as a transcriptional regulator to mediate dioxin toxicity (Esser et al., 2009). AhR activation also results in immunotoxicity. Most studies show that dioxin-mediated activation of AhR suppresses humoral, cellular, and innate immune responses (Esser et al., 2009; Stockinger, 2009). However, some reports showed the immune system could also be activated by dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). For example, stimulated human synoviocytes of rheumatoid arthritis by TCDD induced inflammatory cytokines via its association with AhR (Kobayashi et

al., 2008). Thus, it is thought that the immunotoxic effects mediated by dioxins are dependent on the cell type, species, or the activating ligand.

AhR are expressed by differentiated $CD4^+$ effector T-cell subsets. In particular, interleukin (IL)-17-producing T-cells (T_H17) show high expressions of AhR. Though regulatory T (T_{reg}) cells also express AhR, the levels are extremely low compared to those on T_H17 cells (Esser et al., 2009). Some AhR ligands, like 6-formylindolo-[3,2-β]-carbaxole (FICZ), interfere with T_{reg} cell differentiation, but induce T_H17 cell differentiation (Quintana et al. 2008). Thus, we hypothesized that Yusho patients may show dysregulated T_H17-mediated immune responses. To investigate this, we measured levels of IL-17 and IL-22 secreted by T_H17 cells in the serum of Yusho patients via ELISA. We also measured levels of several cytokines that regulate T_H17 cell proliferation, and other inflammatory cytokines produced by T_H17-stimulated neutrophils and macrophages.

Materials and Methods

Study Protocol

The study protocol was approved by the Japanese Ministry of Health, Labor, and Welfare and all subjects provided informed consent prior to enrolment in the study. In 2004, the Japanese Ministry of Health Labor and Welfare proposed a set of criteria for Yusho patients; these were based on clinical symptoms such as acneiform eruptions, pigmentation, and eyelid swelling, as well as serum high levels of PCBs, PCQs or PCDFs. In our study, blood samples from 40 Yusho patients who met the criteria were obtained in 2005. In parallel, blood samples were obtained from 40 healthy Japanese residents from the same locality of similar age and gender (normal controls). The Yusho patient mean age was 73.6 ± 8.3 years and that of the controls was 69.9 ± 9.8 years. The blood samples of Yusho patients were analyzed for PCBs by saponification in 1 M

NAOH ethanol solution, extraction with *n*-hexane column chromatography on silica gels, and then gas chromatography with electron capture detection (Masuda et al., 1985). The values of PCB obtained represent the concentrations of total coplanar PCBs, and were mainly comprised of 3,3',4,4'-tetra-chlorinated biphenyl (CB), 3,3',4,4',5-penta-CB, and 3,3',4,4',5,5'-hexa-CB in the present study.

Sample Collection

Fresh venous blood samples (8 ml/subject) were collected at 9 AM into serum (silicone-coated) Vacutainer tubes (BD, Fukuoka, Japan). Each sample was allowed to clot and then centrifuged to obtain the serum. All serum samples were stored at -70°C until analyzed. All patients and controls were non-smokers. None of the Yusho patients had been treated with steroids, prostanoids, or other immunosuppressants, and none had a recent history of infection, collagen diseases, or abnormal liver function at the time of sampling.

Measures of Serum Cytokines

Serum IL-17, IL-22, IL-1β, IL-23, and TNFα levels were measured using specific ELISA kits, according to manufacturer instructions (R&D Systems, Minneapolis, MN). Each sample was tested in duplicate. The levels of sensitivity of the purchased IL-17, IL-22, IL-1β, IL-23, and TNFα kits were, respectively, 1.4, 15.0, 4.0, 39.0, and 0.5 pg/ml.

Statistical Analysis

All statistics in the study were performed using Graph Pad Prism® software. A Mann-Whitney U-test was used to compare serum cytokines levels between Yusho patients and controls. In addition, a Spearman's rank correlation was used to analyze correlation between levels of PCBs, PCQs, or PCDFs and serum cytokines levels. The outliers were not eliminated. A p-value < 0.05 was considered statistically significant.

Results

Cytokines Secreted by T_H17 Cells

Serum IL-17 levels were significantly higher in Yusho patients (6.75 [± 6.78] pg/ml) than in controls (1.59 [± 2.99] pg/ml; $p < 0.0001$; Figure 1A). There was a weak negative correlation between IL-17 levels and PCB levels in the serum obtained from Yusho patients ($p < 0.05$, $r = -0.378$; Figure 1B). By contrast, serum IL-22 levels were significantly lower in Yusho patients (29.68 [± 23.04] pg/ml) than in controls (140.59 [± 120.49] pg/ml, $p < 0.0001$; Figure 2). There was no correlation between PCB, PCDF, or PCQ levels and IL-22 levels (data not shown).

Inflammatory Cytokines Possibly Secreted by Neutrophils and Macrophages Activated by T_H17 Cells

Serum IL-1β levels were significantly higher in Yusho patients (14.22 [± 5.16] pg/ml) than in control subjects (9.71 [± 3.95] pg/ml, $p < 0.0001$; Figure 3A). Furthermore, the Yusho patients exhibited significantly elevated serum TNFα levels (10.89 [± 6.98] pg/ml) compared to controls (7.39 [± 3.77] pg/ml, $p < 0.01$; Figure 3B). There was no correlation between PCB, PCDF, or PCQ levels and either levels of IL-1β or TNFα (data not shown).

Cytokines that Regulate T_H17 Cell Proliferation and Differentiation

Serum IL-23 levels were significantly higher in Yusho patients than in controls (76.99 [± 20.14] pg/ml *vs*. 63.71 [± 18.75] pg/ml, respectively, $p < 0.01$; Figure 4).

Discussion

Figure 5 illustrates how cytokines might regulate T_H17 cell differentiation and function. The present study revealed that Yusho patients had higher serum levels of IL-17 than controls. Since IL-17 is the major cytokine secreted by T_H17 cells, these results suggest that T_H17 cells are activated in Yusho patients. Furthermore, as IL-17 activates both macrophages and neutrophils

(Katz et al., 2001; Barin et al., 2012), we also measured the cytokines secreted by these cell types. Serum IL-1β and TNFα levels were higher in Yusho patients than in controls, indicating a presence of activated macrophages and neutrophils. It is surprising that active inflammatory cytokines production was observed in Yusho patients almost 40 years after the initial poisoning by PCBs or PCDFs. Dioxin-like components, once released into the circulation little by little from reservoirs such as fat stores, may perturb the host immune system. Moreover, it may be related to the high levels of IL-1β and IL-23, both of which regulate T_H17 cell proliferation and differentiation (Kurebayashi et al., 2013). Overall, these results suggested that T_H17 cells are activated in Yusho patients.

Interestingly, another study showed that rheumatoid factor levels were higher in the serum of Yusho patients than in those of controls (Nagayama et al., 2001). As activated T_H17 cells play a key role in rheumatoid arthritis (Maddur et al., 2012), Yusho patients should be monitored for rheumatoid arthritis. Moreover, systemic lupus erythematosus, inflammatory bowel diseases, or psoriasis vulgaris, pathologies in which activated T_H17 cells also play a key role, should be also mentioned in Yusho patients.

Precise mechanisms underlying T_H17 cell activation in Yusho patients are unclear. In mice models, activated T_H17 cells also express high levels of AhR (Quintana et al., 2008). FICZ, a tryptophan product and not a dioxin, has been shown to act as an endogenous AhR ligand that induced T_H17 cell differentiation and activation when present with certain combinations of cytokines (Quintana et al., 2008). Accordingly, taken together with our results, PCBs, PCQs, and/or PCDFs may act as AhR ligands and play a role in driving T_H17 cell proliferation and activation in Yusho patients. Quintana et al. (2008) also showed that TCDD, a different type of dioxin, could induce differentiation and activation of T_{reg} cells, but not T_H17 cells; this outcome

was likely opposite to the dioxins-like components' effects on T_H17 cells in Yusho patients. There is a report of a dioxin and dioxin-like species-specific activation of AhR *in vitro*. For example, in Panc1 and HEK293 cell lines, TCDD induced higher levels of AhR-related gene-expression, such as that of CYP1A1 than PCB126. In contrast, PCB126 showed more enhanced induction of AhR-related genes than did TCDD in Hepa1c1c7 cell lines (Zhang et al., 2008). Thus, there seems to be a difference in AhR activation among the various types of dioxins and dioxin-like components. Further study, such as *in vitro* analysis of T_H17 cell differentiation induced by PCBs, PCQs, or PCDFs would likely help to clarify the relationship between dioxin species and T-cell activation in Yusho patients.

Meanwhile, as with TCDD, it could be hypothesized that PCBs, PCDFs, and/or PCQs induced T_{reg} cell differentiation in Yusho patients. Recent studies revealed IL-17-producing $FOXP3^+$ regulatory T (Tr17) cells. Tr17 cells express Foxp3 and display immunosuppressive function, while participate in innate immunity by producing IL-17 as well (Voo et al., 2009; Li et al., 2012). Tr17 cells have been identified in human peripheral blood and lymphoid tissue (Voo et al., 2009). Moreover, it was shown that they were differentiated from $CD4^+FOXP3^+$ T_{reg} cells when cultured with antigen presenting cells and cytokines such as IL-1β, IL-2, IL-21 and IL-23 (Voo et al., 2009; Koenen et al., 2008). Taken together with our results that IL-1β and IL-23 levels were elevated in Yusho patients, PCBs, PCDFs, and/or PCQs might have induced Tr17 cells in these individuals.

In this study, serum IL-22 levels were lower in Yusho patients than in controls. This result was not accordance with results expected from simple T_H17 cell activation, because activated T_H17 cells produce both IL-22 as well as IL-17 (Zenewicz and Flavell, 2011). However, production of IL-17 and IL-22 seems not to be strictly coordinated and rather depends on species

of stimuli, cell type, or pathways induced. For example, human T_H17 cells that were stimulated by IL-23 and prostaglandin E2 displayed enhanced IL-17 production but decreased IL-22 production (Chizzolini et al., 2008). It was also seen that human $CD4^+$ T-cells when activated by TCDD produced high levels of IL-22 but not IL-17 (Ramirez et al., 2010). As for the results here, unlike what was seen with TCDD *in vitro*, the PCBs, PCDFs, PCQs possibly could have induced an up-regulation of IL-17 and down-regulation of IL-22 formation in the Yusho patients. As such, the precise mechanism of regulation of IL-17 and IL-22 in Yusho patients remains uncertain; however, *in vitro* studies with PCBs, PCQs, and/or PCDFs could help reveal the mechanism underlying $CD4^+$ T-cell activation and differentiation in Yusho patients.

Conclusion

The results of the present study suggested to us that T_H17 cells mediate inflammatory responses in Yusho patients. However, this study was small-sized study. Further large-scale studies of the immune responses in the Yusho patients are required.

Acknowledgement

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure Legends

Figure 1. Serum IL-17 levels. (A) IL-17 levels in serum samples obtained from Yusho patients and controls. Serum IL-17 levels were significantly higher in the Yusho patients ($p < 0.0001$). **(B)** A weak negative correlation was observed between IL-17 levels and PCB levels in the sera of Yusho patients ($p < 0.05$, $r = -0.378$). The values of PCBs obtained represent the concentra- tions of total coplanar PCBs that are mainly of 3,3',4,4'-tetra-chlorinated biphenyl (CB), 3,3',4,4',5-penta-CB, and 3,3',4,4',5,5'-hexa-CB in the present study.

Figure 2. Serum IL-22 levels. Serum IL-22 levels were significantly lower in Yusho patients than in controls ($p < 0.0001$).

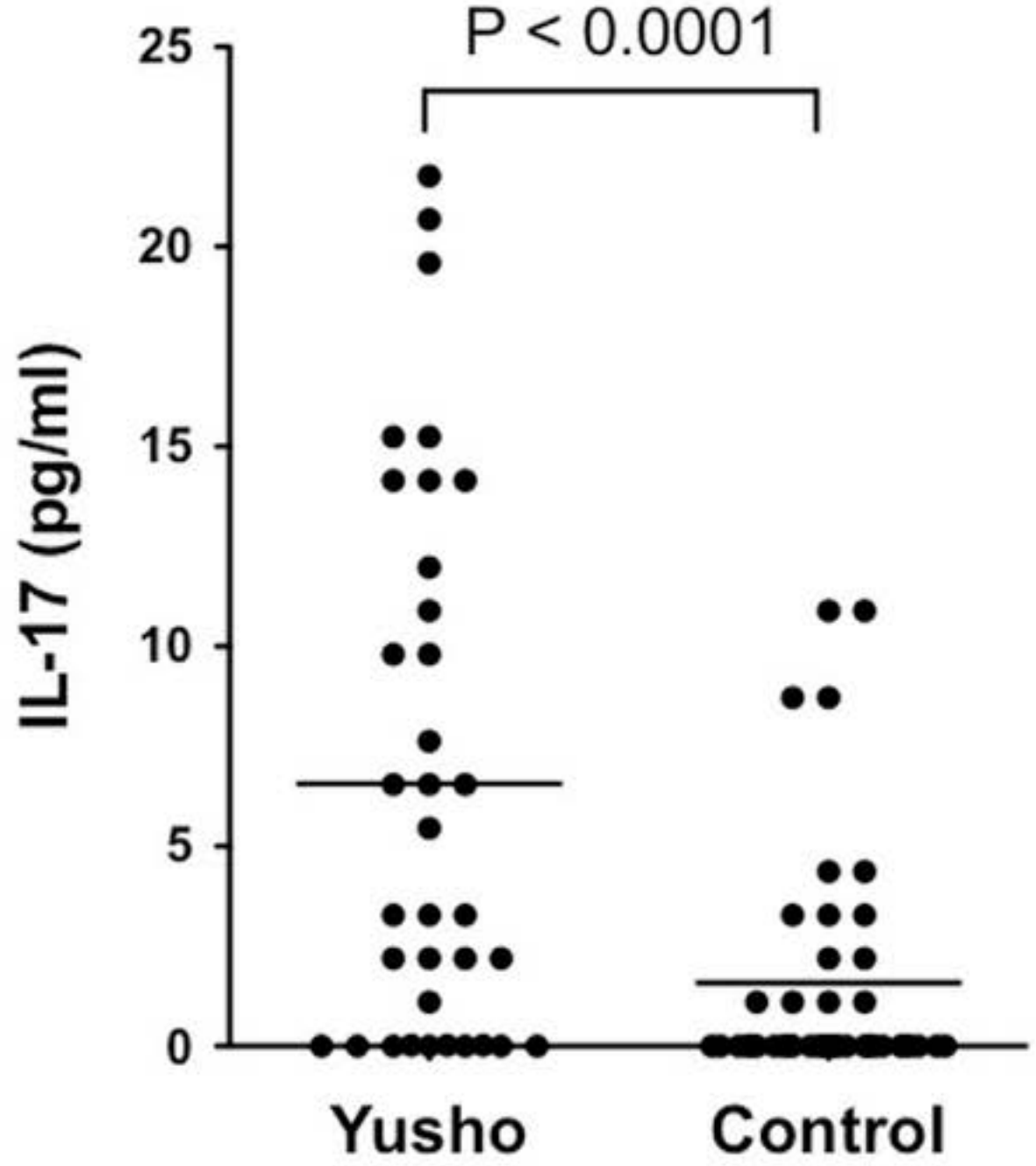
Figure 3. Serum IL-1β and TNFα levels. Serum **(A)** IL-1β and **(B)** TNFα levels were significantly higher in Yusho patients than in controls ($p < 0.0001$ and $p < 0.01$, respectively).

Figure 4. Serum IL-23 levels. Serum IL-23 levels were significantly higher in Yusho patients than in controls ($p < 0.01$).

Figure 5. A proposed mechanism of T_H17 cell activation and subsequent inflammatory responses in Yusho patients. Yusho patients ingested several dioxins such as PCBs or PCDFs in contaminated rice oil that then acted as ligands for AhR expressed by T_H17 cells. Binding to AhR may have activated T_H17 cells, which then secreted large amounts of IL-17 that stimulated neutrophils and macro- phages that, in turn, secreted IL-1β and TNFα to induce inflammatory responses. High levels of IL-1β and IL-23 induce T_H17 cell differentiation from T_H0 cells. However, the T_H17 cells in Yusho patients might secrete IL-17 but not IL-22. Cytokines measured in this study are in bold. Neut: neutrophil, MΦ: macrophage.

Figure 1

A.


P < 0.0001
IL-17 (pg/ml)
25
20
15
10
5
0
Yusho
Control

B.

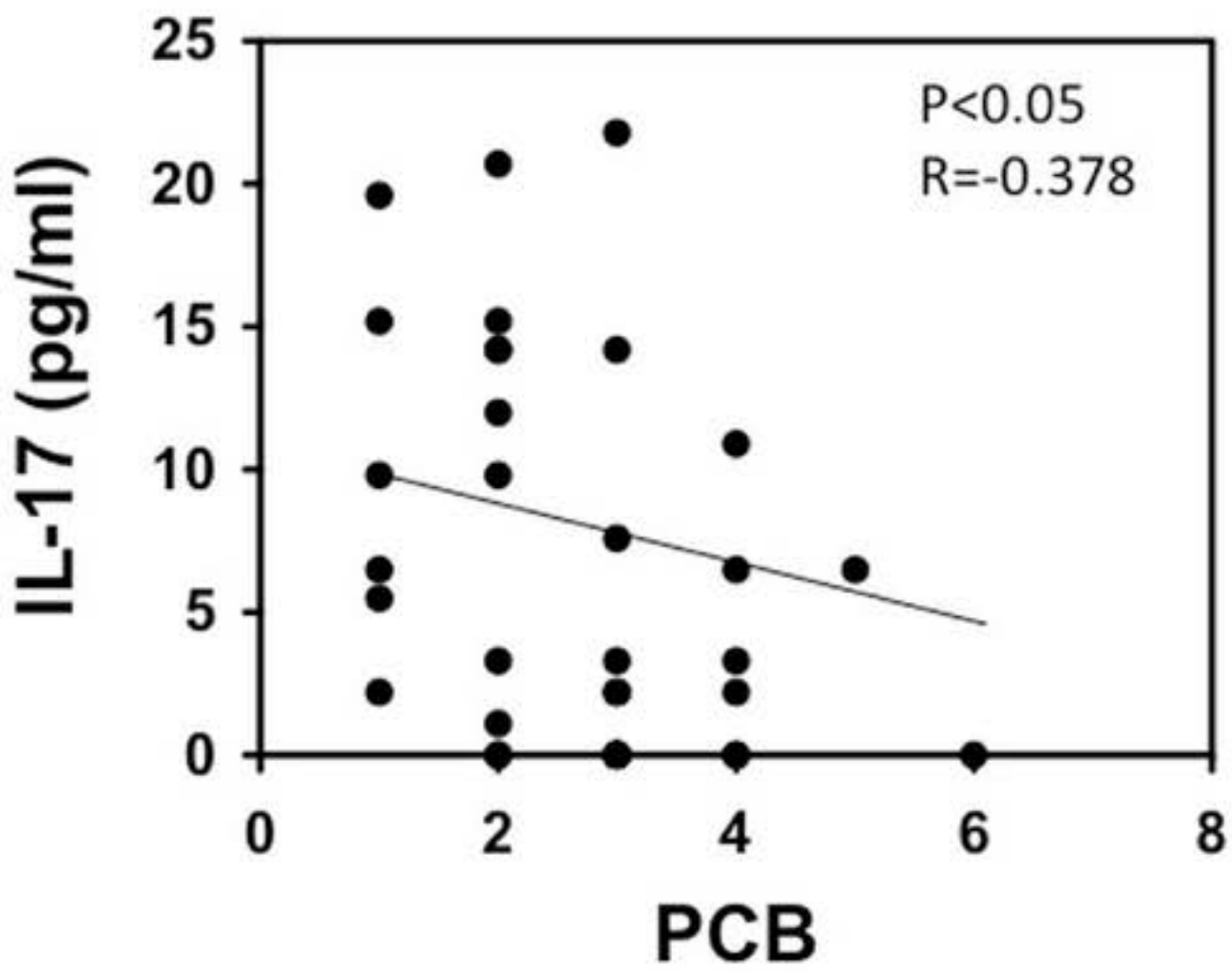

P<0.05
R=-0.378
IL-17 (pg/ml)
25
20
15
10
5
0
0
2
4
6
8
PCB

Figure 2

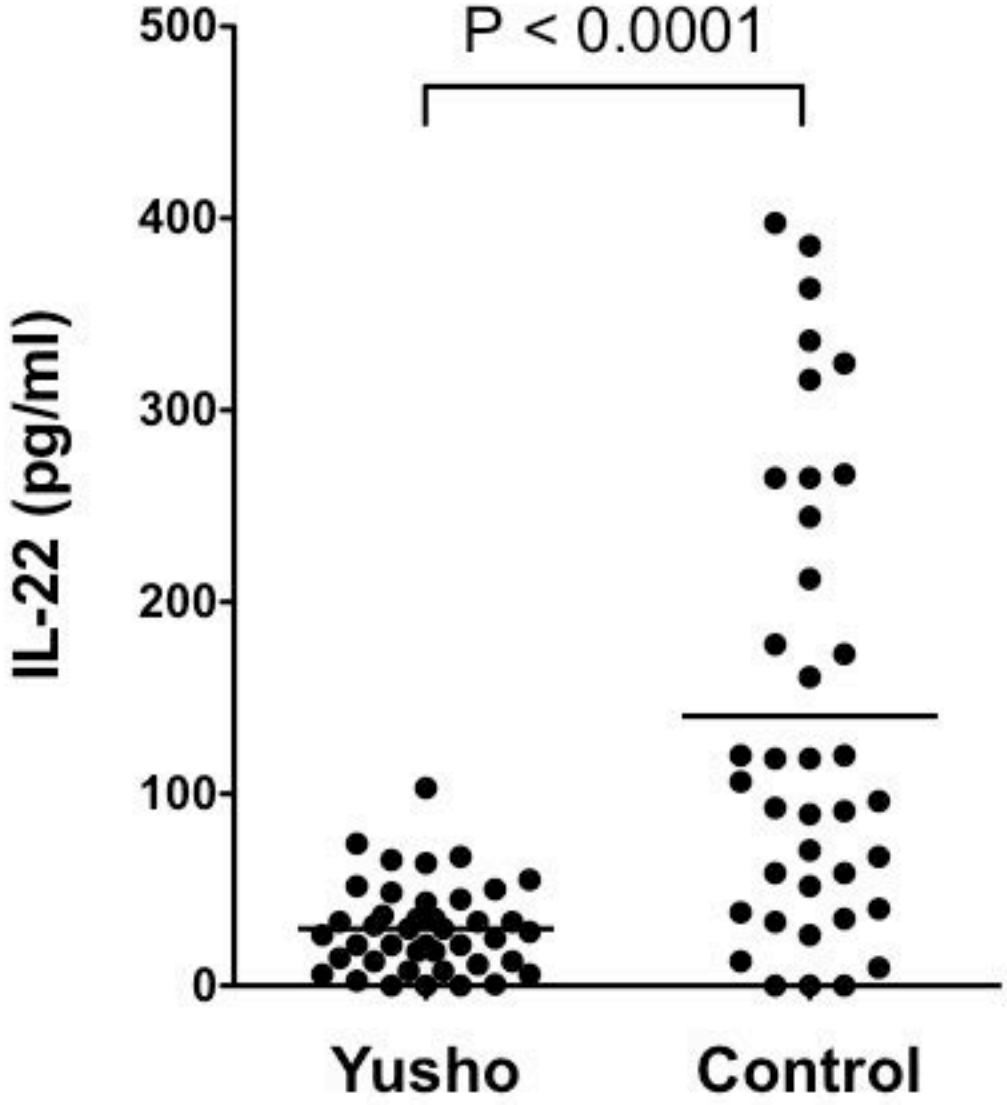
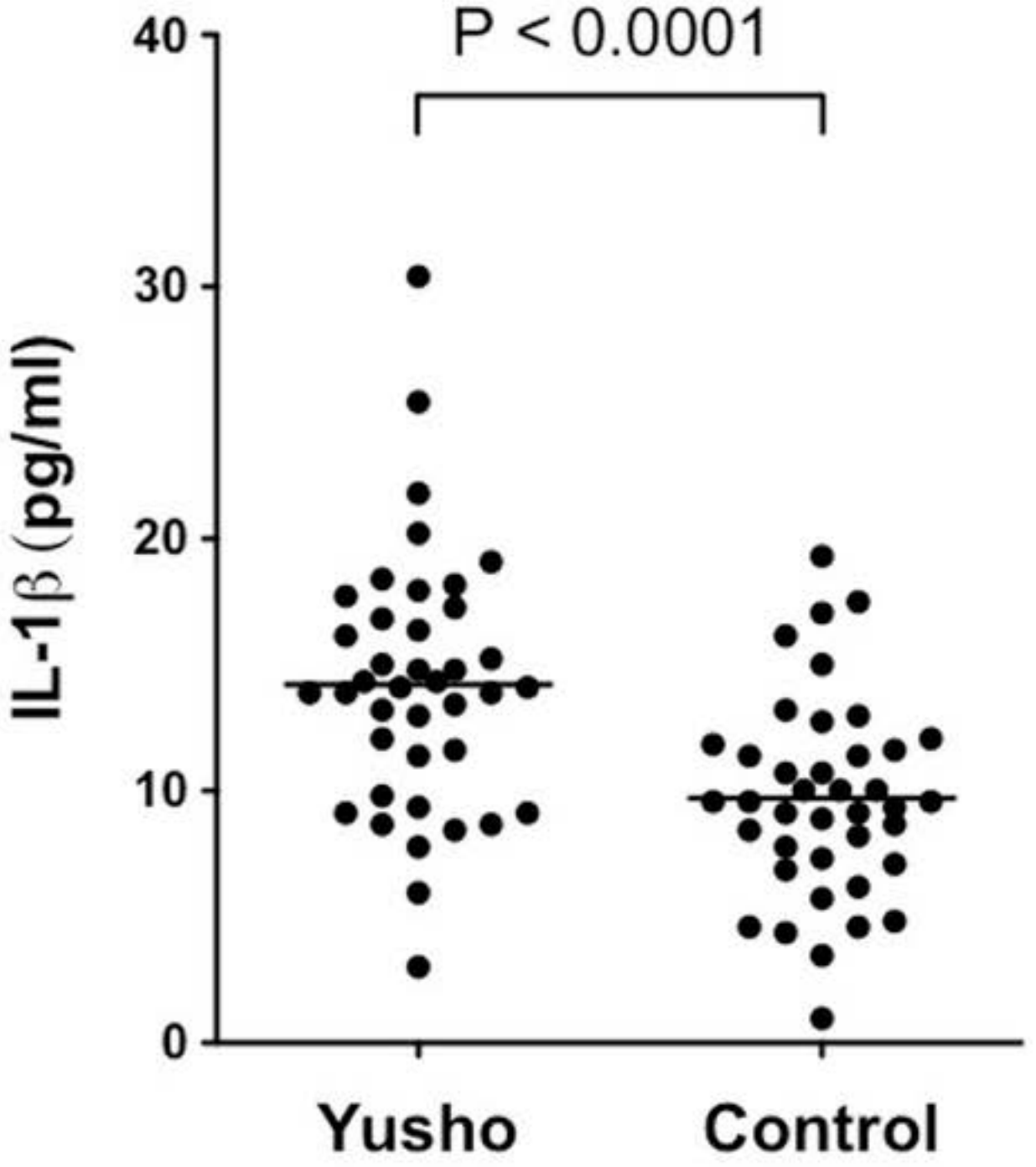


Figure 3

A.


P < 0.0001
IL-1β (pg/ml)
40
30
20
10
0
Yusho
Control

B.

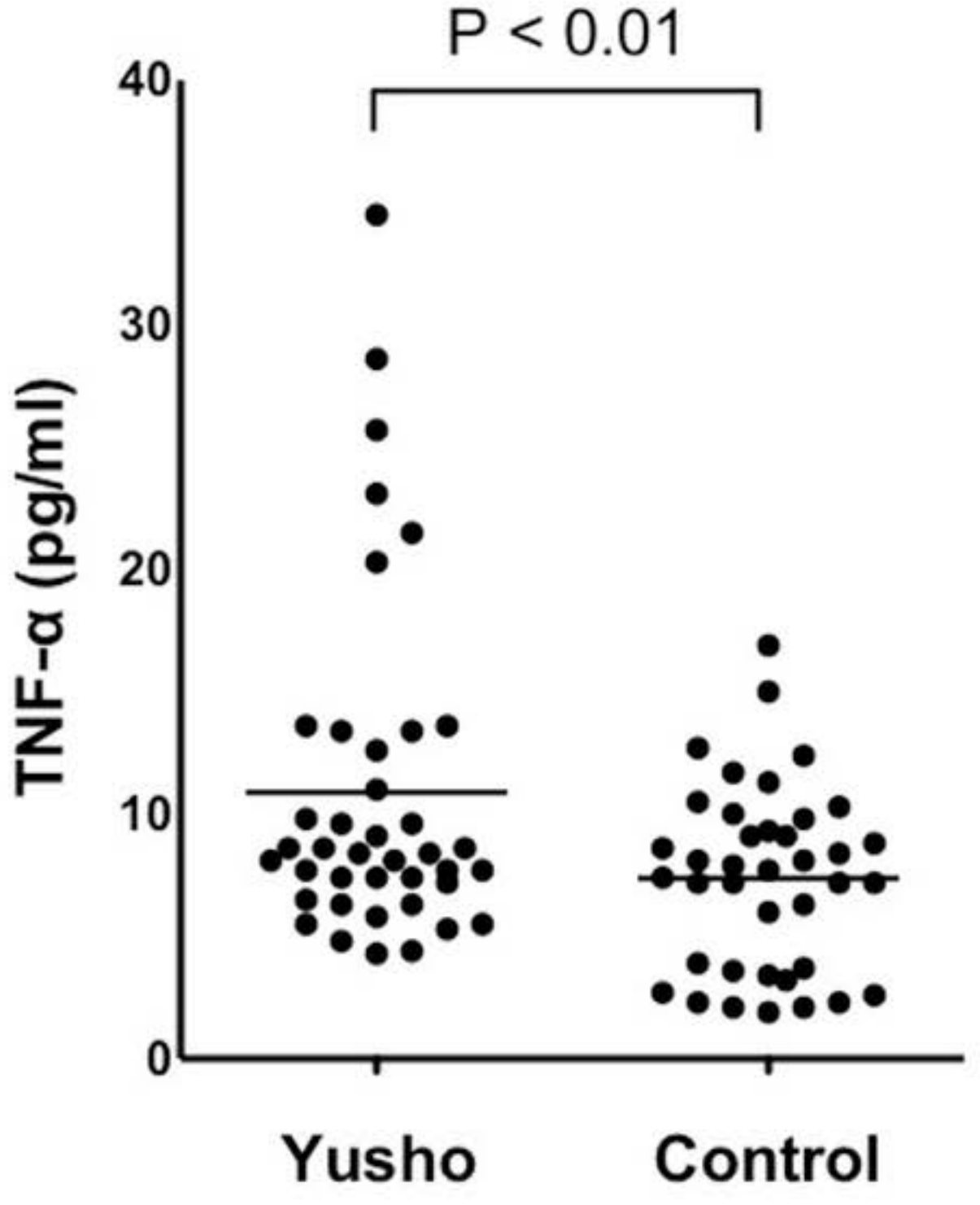

P < 0.01
TNF-α (pg/ml)
40
30
20
10
0
Yusho
Control

Figure 4

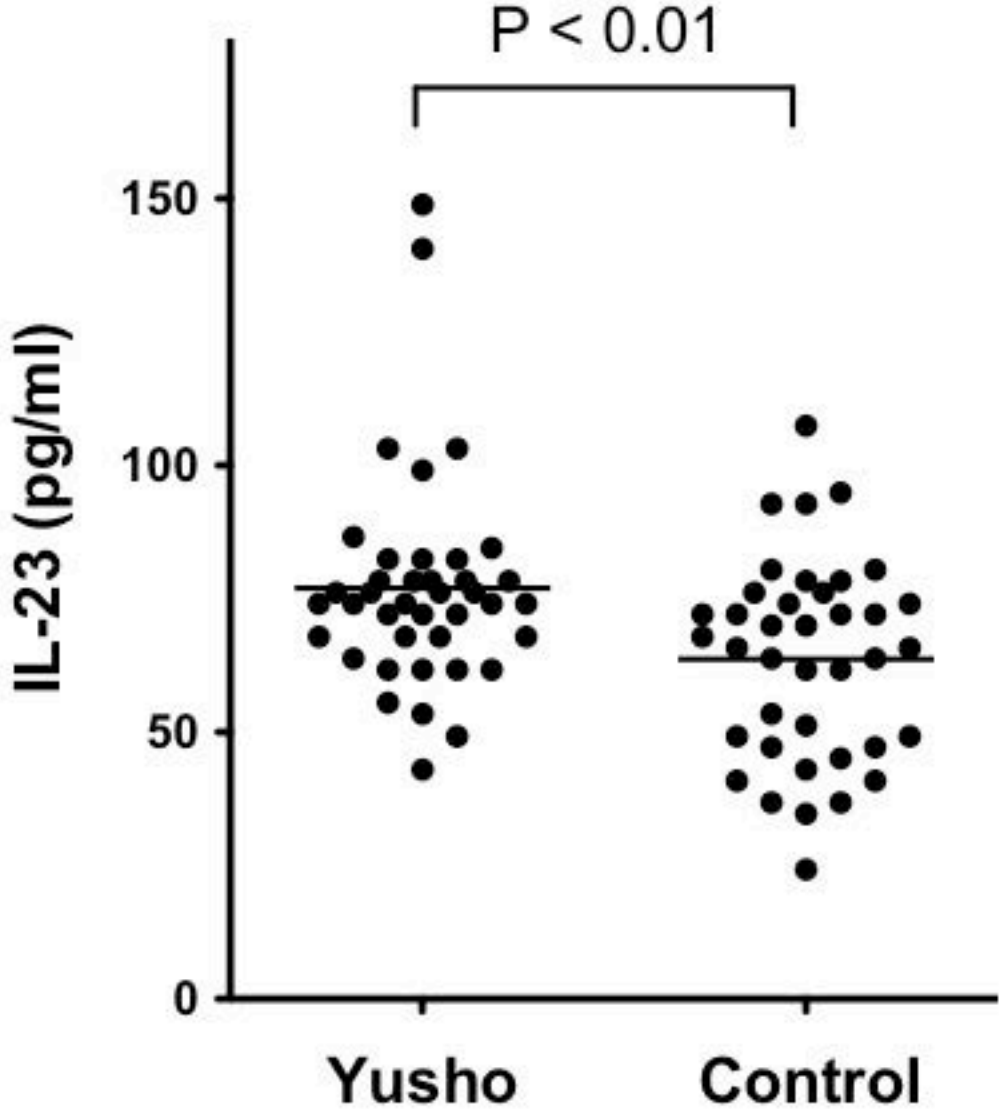
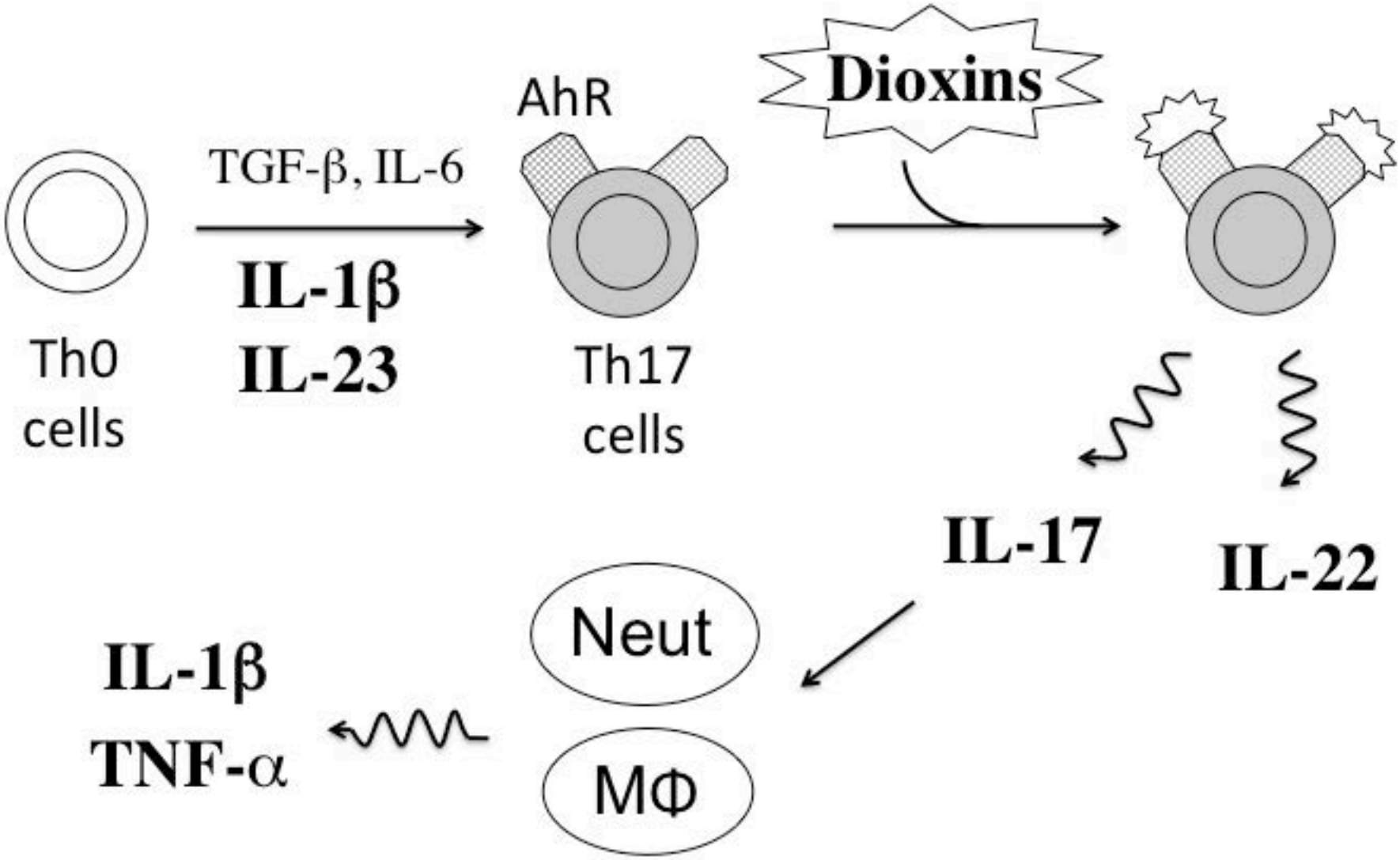


Figure 5


AhR
Dioxins
TGF-β, IL-6
IL-1β
IL-23
Th0 cells
Th17 cells
IL-17
IL-22
Neut
MΦ
IL-1β
TNF-α