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Insight into innate immune response in "Yusho": The impact of natural killer cell and regulatory T cell on inflammatory prone diathesis of Yusho patients

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Conflict of interest and ethics statement

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Abstract

Background: In 1968 in western Japan, polychlorinated biphenyl-contaminated "Kanemi rice oil" was used in cooking, causing food poisoning in many people. More than 50 years have passed since the Yusho incident, and although inflammatory disorders such as suppuration have been observed in Yusho patients, the etiology of this inflammation susceptibility remains obscure.

Objectives: To investigate the mechanisms of susceptibility to inflammation in Yusho patients, peripheral immune cell fractions and concentrations of inflammatory cytokines were evaluated in blood samples collected from both Yusho patients and age-matched healthy subjects undergoing medical examination in Nagasaki.

Methods: To exclude diagnostic uncertainty, serum levels of polychlorinated biphenyl (PCB), polychlorinated quarterphenyl (PCQ), and polychlorinated dibenzofuran (PCDF) were measured. Immune cell (e.g.. natural killer and regulatory T cell) populations were analyzed by flow cytometry. Serum cytokines involved in immune cell activation were measured by ELISA.

Results: The relative proportion of natural killer cells was higher in Yusho patients than in healthy subjects, while the proportion of regulatory T cells did not differ between groups. Serum concentrations of IL-36 and IFN-γ were significantly lower in Yusho patients than in healthy subjects. Conversely, serum cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), which is a cytokine related to activated NK cells, was higher in Yusho patients than in healthy subjects and was positively correlated with PCDF blood levels.

Conclusion: Increased numbers of NK cells in Yusho patients suggests that the innate immune response has been activated in Yusho patients. The seemingly paradoxical results for CTLA-4 and IFN-γ may reflect counterbalancing mechanisms preventing excessive NK cell activation. This dysregulation of innate immunity might contribute to the inflammation observed in Yusho patients.

Abbreviations

CTLA, cytotoxic T lymphocyte-associated antigen; IFN, interferon; IL, interleukin; PCB, polychlorinated biphenyl; PCDF, polychlorinated dibenzofuran; PCQ, polychlorinated quarterphenyl; SD, standard deviation; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TNF, tumor necrosis factor.

Keywords

Dioxins; Yusho; natural killer cells; suppuration; innate immunity; cytokines.

1. Introduction

Fifty years have elapsed since the occurrence of the Yusho incident in 1968 in western Japan (Kuratsune et al., 1972). Yusho was caused by ingesting rice bran oil containing dioxins, including mixed polychlorinated biphenyl (PCB), polychlorinated quarterphenyl (PCQ), and polychlorinated dibenzofuran (PCDF). Diagnostic criteria of Yusho is composed by characteristic signs and blood dioxin level. Clinical sings are acne-like eruption, pigmentation of skin and mucosa, and hypersecretion of Meibomian gland. Furthermore, Yusho patients meet any of the following blood dioxin level, (1) aberrant level of PCB (no provided criteria value), (2) increased PCQ level (>0.1 ppb), or (3) increased PCDF level (>50pg/g lipids). Although blood concentrations of dioxins in Yusho patients are gradually decreasing, they remain higher than that of control subjects. The cumulative number of Yusho patients is over 2,300, and the exact number of survivors is still missing. Early intense symptoms have tended to regress; however, Yusho patients continue to experience relapses of acne-like skin lesions and respiratory symptoms, such as cough and sputum.

Since the Yusho incident, medical examinations and questionnaire surveys have been conducted. Based on these surveys, Yusho patients were noted to have higher rates of atheroma, acne, and eczema (18.7%, 35.5%, and 39.4%, respectively) than healthy controls (2.1%, 13.0%, and 10.8%, respectively) (Akahane et al., 2018). Thus, confirmed Yusho patients appear to remain susceptible to inflammatory conditions.

The aryl hydrocarbon receptor (AhR) is a receptor for these polycyclic aromatic hydrocarbons and dioxin-like congeners (Esser et al., 2009). AHR is expressed on various immune cells, including T cells, B cells, neutrophils, macrophages, dendritic cells, and innate lymphoid cells (Kreitinger et al., 2016). However, it has not been fully understood how dioxin-like compounds (e.g., PCB, PCQ, and PCDF) affect immune cells. In addition to cytokine modulation including interleukin (IL)-1β, IL-6 and IL-8 (Kobayashi et al., 2008), recent studies stress a pivotal role of AHR in the regulation of natural killer (NK) cells and regulatory T (Treg) cells (Ambrosio et al., 2019; Kreitinger et al., 2016; Shin et al., 2013; Stockinger et al., 2014). Our previous report demonstrated that levels of various serum cytokines, such as IL-17, IL-23, IL-1β and tumor necrosis factor-α (TNF-α), are increased in Yusho patients (Kuwatsuka et al., 2014). IL-1, IL-23, and IL-17 involves in the immune responses to bacterial infection via activation of NK cells (Doisne et al., 2011) (Cella et al., 2010). Furthermore, increased serum levels of IL-10 and IL-35, which are derived mainly from Treg cells, have been observed in Yusho patients (Koike et al., 2013). These results prompted us to hypothesize that NK cells and Treg cells might be

involved in the pathogenesis of pro-inflammation in Yusho disease.

In this study, we examined the features of both immune cells and inflammatory cytokines in Yusho patients and considered their implications with respect to the etiology of inflammation susceptibility observed in these individuals.

2. Materials and methods

2.1 Study protocol

The study protocol was approved by the Japanese Ministry of Health, Labor, and Welfare and the Kyushu University Institutional Review Board for Clinical Research (approval no. 30-384). All subjects provided informed consent prior to enrollment in the study. At the time of an annual medical examination in Nagasaki prefecture, we obtained blood samples from 31 Yusho patients and 31 healthy Japanese volunteers (controls), who were matched for age and sex and from the same locality. Mean (± standard deviation [SD]) age was 72.0 ± 8.6 years for the Yusho patients and 67.9 ± 11.0 years for the controls. These blood samples were used for cytokine testing. However, due to lack of serum sample, CTLA-4 was measured using samples that are partially different from the samples that measured other cytokines. Patients' serum samples used in CTLA-4 measurement were collected from same cohort as other cytokine testing. Mean (± standard deviation [SD]) age was 71.4± 6.4 years for the Yusho patients and 70.4 ± 8.5 years for the controls.

Blood for flow cytometry was obtained on a different day from the blood cytokine measurements. Therefore, blood for flow cytometry was collected from Yusho patients belonging same cohort as cytokine testing. Yusho patients who agreed to return for blood sampling on a separate day from the medical examination. Because of this, the number of subjects who underwent flow cytometry differed from the number who underwent cytokine testing: 53 Yusho patients and 14 controls. The mean age of patients who underwent flow cytometry was likewise different from the above values: 67.2 ± 12.1 years for the Yusho patients and 70.6 ± 10.3 years for the controls.

The blood concentrations of PCB (ppb), PCQ (ppb) and PCDF (pg/g lipids) were measured by using high-resolution gas chromatography/high-resolution mass spectrometry as previously reported (Todaka et al., 2008; Yasutake et al., 2011).

2.2 Flow cytometry analysis

Populations of lymphocyte subsets were analyzed by flow cytometry using a BD FACSCanto II (Becton Dickinson [BD], San Jose, CA, USA). Populations of CD3-

positive T cells, CD4-positive T cells, CD8-positive T cells, and NK cells were detected using BD Multitest 6-Col TBNK reagent (Cat No. 044611, BD Biosciences, San Jose, CA, USA). Populations of Treg cells were detected with CD4 antibody (BD human CD4 PERCP mAb [No. 347324], BD Biosciences), CD25 antibody (BD human CD25 APC mAb [No. 347324], BD Biosciences), and CD127 antibody (BD FITC mouse anti-human CD127 [No. 560549], BD Biosciences). Samples were prepared and analyzed according to the manufacturer's instructions. Data were analyzed using BD FACSDiva™ (BD Biosciences) software.

2.3 Measurement of serum cytokines

Serum concentrations of interferon (IFN)-γ, IL-2, and IL-33 were measured using the corresponding human ELISA kits, according to manufacturer instructions (Cat. No. DIF50, RS120, D3300, respectively, R&D Systems, Minneapolis, MN, USA). Serum cytotoxic T lymphocyte-associated antigen (CTLA)-4 was measured using a specific human ELISA kit, according to the manufacturer's instructions (eBioscience). Serum IL-36γ were measured using a specific human ELISA kit, according to the manufacturer's instructions (Cat. No. CSB-EL011620HU, CUSABIO, Houston, TX, USA). Absorbance was determined using Multiskan MS (Thermo Fisher Scientific, Tokyo, Japan).

2.4 Statistical analysis

Statistical analysis was performed using Graph Pad Prism® software (GraphPad Software, San Diego, CA, USA). Unpaired t-test with Welch's correction was used to compare serum cytokine levels between Yusho patients and controls. Spearman's rank correlation analysis was used to assess relationships between PCB, PCQ, and PCDF levels and serum cytokine levels. Outliers were not removed. P-values <0.05 were considered statistically significant.

3. Results

3.1 Proportions of Treg and NK cells in Yusho patients

To examine the immunological features of Yusho patients, we analyzed the proportions of lymphocyte subsets, especially Treg and NK cells. We found that the proportion of Treg cells was not significantly different between Yusho patients (mean ± SD: 7.9 ± 3.4%) and healthy controls (mean ± SD: 7.2 ± 2.4%) (p=0.4484) (Fig. 1a). By contrast, the proportion of NK cells was significantly higher in Yusho patients (mean ± SD: 21.8 ± 9.4%) than in controls (mean ± SD: 16.6 ± 7.8%) (p=0.0442) (Fig. 1b).

3.2 Serum levels of cytokines related to NK and Treg cells

To investigate factors associated with proneness to inflammation and activity of both NK cells and Tregs, we next measured serum levels of various cytokines, including IFN-γ, CTLA4, IL-2, IL-33, and IL-36γ.

The serum concentration of IFN-γ, which is released from activated NK cells, was significantly lower in Yusho patients (mean ± SD: 61.8 ± 8.6 ng/mL) than in controls (mean ± SD: 78.7 ± 11.0 ng/mL) (p=0.0049) (Fig. 2a). The serum concentration of CTLA-4, which is a key molecule involved in immunologic self-tolerance via Treg activation, was significantly higher in Yusho patients (mean ± SD: 18.2 ± 12.4 pg/mL) than in controls (mean ± SD: 12.4 ± 8.0 pg/mL) (p=0.0381) (Fig. 2b). Serum concentrations of IL-2 and IL-33, which are potent stimulators of NK cells (Liew et al., 2010) (Vacca et al., 2019), were comparable between Yusho patients (mean ± SD: 8.8 ± 1.2 pg/mL and 76.42 ± 30.0 pg/mL, respectively) and controls (mean ± SD: 6.8 ± 0.7 pg/mL and 69.7 ± 36.5 pg/mL, respectively) (Fig. 2c,2d).

In contrast to the above cytokines, the serum concentration of IL-36γ, which negatively regulates NK cell activation (Mele et al., 2017), was significantly lower in Yusho patients (mean ± SD: 255.3 ± 206.9 pg/mL) than in controls (mean ± SD: 639.4 ± 701.2 pg/mL) (p=0.0060) (Fig. 2e).

Differences in cytokine levels between individuals are shown graphically in Supplemental Figure 1. No obvious trends were observed between individuals for any of the cytokine levels.

3.3 Correlations between dioxins and cytokines

In Yusho patients, serum levels of IFN-γ, IL-2, IL-33, and IL-36γ did not correlate with blood concentrations of any of the three measured dioxins (PCDF, PCB, and PCQ) (Figure 3, a). CTLA-4 was positively correlated with the PCDF level (p=0.0302, r=0.4030), but not with PCQ or PCB levels (Fig. 3, a, b).

4. Discussion

This study was conducted to assess the immunological features of Yusho patients, with special emphasis on the activities of NK and Treg cells. To date, very little published research has examined the impact of orally ingested dioxins on human immune cells.

In a previous study of murine NK cells, activation of AhR using the endogenous tryptophan derivative 6-formylindolo[3,2-b]carbazole (FICZ) promoted IFN-γ production, whereas AhR-deficient NK cells had reduced cytolytic activity (Shin et al.,

2013). Conversely, some previous studies reported that AhR activation suppressed NK cell activation (Wang et al., 2009). In the current study, the proportion of NK cells was significantly higher in Yusho patients than in healthy controls, which contrasts with the results of a study of adolescents in Flanders, which found that the percentage of NK cells in blood was negatively correlated with serum PCB concentration (Van Den Heuvel et al., 2002). The discrepancy between study results may relate to differences in dioxin exposure. In the Flanders study, the subjects were naturally exposed to dioxins, whereas in our study, Yusho patients ingested high level of dioxins. Regulation of NK cell populations might differ depending on the amount of dioxin exposure.

To evaluate the degree of NK cell activity, we measured serum concentrations of several cytokines. Although IL-2 level failed to differ between Yusho patients and healthy controls, significant differences were found for IFN-γ and IL-36γ. Unexpectedly, serum IFN-γ was significantly decreased in Yusho patients. As mentioned above, activated NK cells are known to produce IFN-γ, and FICZ, an endogenous agonist for AhR, can activate NK cells to produce IFN-γ (Shin et al., 2013). Our results suggest that NK cells in Yusho patients are increased in number but not exhausted. Another possibility is that the differences in IFN-γ production by NK cells might reflect differences between exogenous or endogenous dioxin exposure. Moreover, it could be speculated that long-term exposure to exogenous dioxins might increase the number of resting NK cells and enhance inflammation susceptibility. IL-36 is a cytokine belonging to the IL-1 family, which plays a role in cutaneous chronic inflammatory responses (Tortola et al., 2012) (Furue et al., 2018) and is known to suppress NK cell activity (Mele et al., 2017). We found that serum IL-36 concentrations were significantly lower in Yusho patients than in controls, consistent with underlying activation of NK cells. Conversely, serum levels of IL-33, an NK cell activator, were comparable between Yusho patients and controls. A recent study reported that the AhR agonist TCDD stimulated IL-33 production in macrophages (Ishihara et al., 2019). Although the above results may seem contradictory with respect to the activity of NK cells, these cytokines may represent counterbalancing mechanisms acting to avoid excessive NK cell activations. Further studies are required to more fully explore this issue.

The increased serum levels of IL-10 and IL-35 in Yusho patients led us to suspect that Treg cells might be involved in the pathogenesis of Yusho disease (Koike et al., 2013). Indeed, several previous studies reported relationships between dioxins and Treg cells. For instance, administration of TCDD induces Treg cell activation in mice, possibly via AhR on T cells (Quintana et al., 2008) (Funatake et al., 2005) (Li et al., 2016). Although we found no increase in Treg cells in Yusho patients, we did observe

significantly higher serum levels of CTLA-4 in these patients, compared with controls. This suggests that Treg cells may be activated in Yusho patients despite no changes in the quantity of Treg cells. Future studies are required to determine whether Treg cells have immuno-effector or immuno-suppressor functions in Yusho patients.

When we assessed the relationships between serum concentrations of cytokines and dioxins, the only significant correlation was a positive correlation between serum PCDF and CTLA4 levels. The other cytokines (IFN-γ, IL-2, IL-33, and IL-36γ) were not correlated with any dioxin (PCB, PCQ, or PCDF), and CTLA4 was not correlated with PCB or PCQ. These results suggest that ingested dioxin, especially PCDF, might affect the immune response. No previous literature reported the relationship between these cytokines and ingested dioxins. The limitation in this study was that we measured the immune cells and cytokines only once. Repeated measurements are warranted to ensure the reproducibility. However, the blood levels of dioxin-like compounds remain stably higher compared with healthy individuals (Matsumoto et al., 2016), we believe that the present data represents the current immune status of Yusho patients.

5. Conclusion

Our findings suggest that disruption of the normal balance between activation of NK cells and Treg cells, as reflected by aberrant cytokine profiles, may explain the increased susceptibility to inflammation in Yusho patients. The results of this study may aid in the development of novel therapeutic interventions for these patients.

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Figure Legends

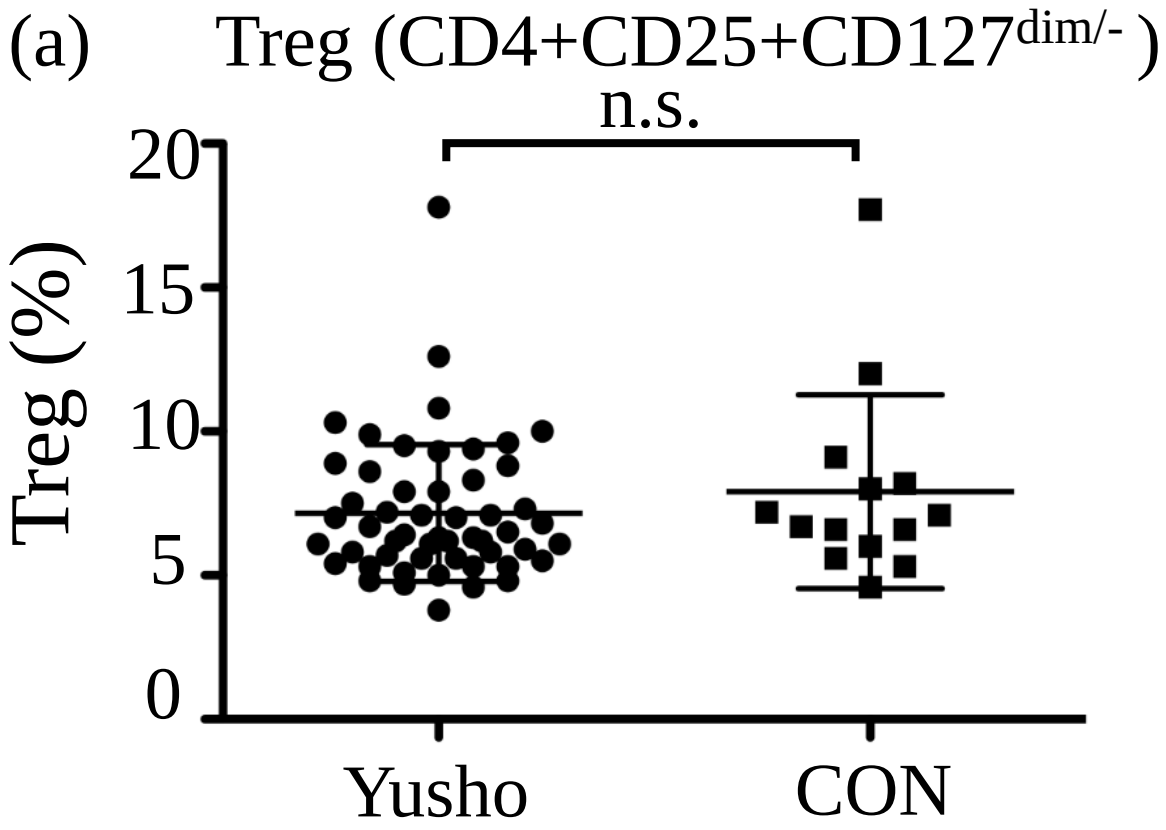
Figure 1. Flow cytometry analysis of Treg and NK cell populations. (a) The proportion of Treg cells was not significantly different between Yusho patients (n=53) and controls (n=31). (b) The proportion of NK cells was significantly higher in Yusho patients (n=53) than in controls (n=31) ($p<0.05$). Yusho: Yusho patients. CON: controls. *:$p<0.05$. n.s.: not significant. Horizontal bar indicates mean value. Error bars represent standard deviation of the mean.

Figure 2. Comparison of serum cytokine levels between Yusho patients and healthy controls. (a) The serum concentration of IFN-γ was significantly lower in Yusho patients (n=31) than in controls (n=31) ($p<0.05$). (b) The serum concentration of CTLA-4 was significantly higher in Yusho patients (n=26) than in controls (n=28) ($p=0.0381$). (c) The serum concentration of IL-2 was not significantly different between Yusho patients (n=31) and controls (n=30) ($p=0.3906$). (d) The serum concentration of IL-33 was not significantly different between Yusho patients (n=31) and controls (n=30) ($p=0.1550$). (e) The serum concentration of IL-36 was significantly lower in Yusho patients (n=31) than in controls (n=30) ($p<0.05$). Yusho: Yusho patients. CON: controls. *:$p<0.05$. n.s.: not significant. Horizontal bar indicates mean value. Error bars represent standard deviation of the mean.

Figure 3. Correlations between serum PCDF, PCQ, or PCB levels and IFN-γ, CTLA-4, IL-2, IL-33, or IL-36 levels in Yusho patients. (a) No significant correlations were observed between serum IFN-γ, IL-2, IL-33, IL-36 levels, and PCDF, PCQ, or PCB levels in Yusho patients. *:$p<0.05$ (also appeared as bold face). (a, b) A positive correlation was observed between serum CTLA-4 level and PCDF level ($p<0.05$, n=24).

Supplemental Figure 1. Differences in cytokine levels between individuals. Yellow: IFN-γ. Dark blue: CTLA-4. Gray: IL-2. Orange: IL-33. Right blue: IL36. The y-axis is displayed on a logarithmic scale.

Figure.1


(a)
Treg (CD4+CD25+CD127dim/-)
n.s.
Treg (%)
20
15
10
5
0
Yusho
CON

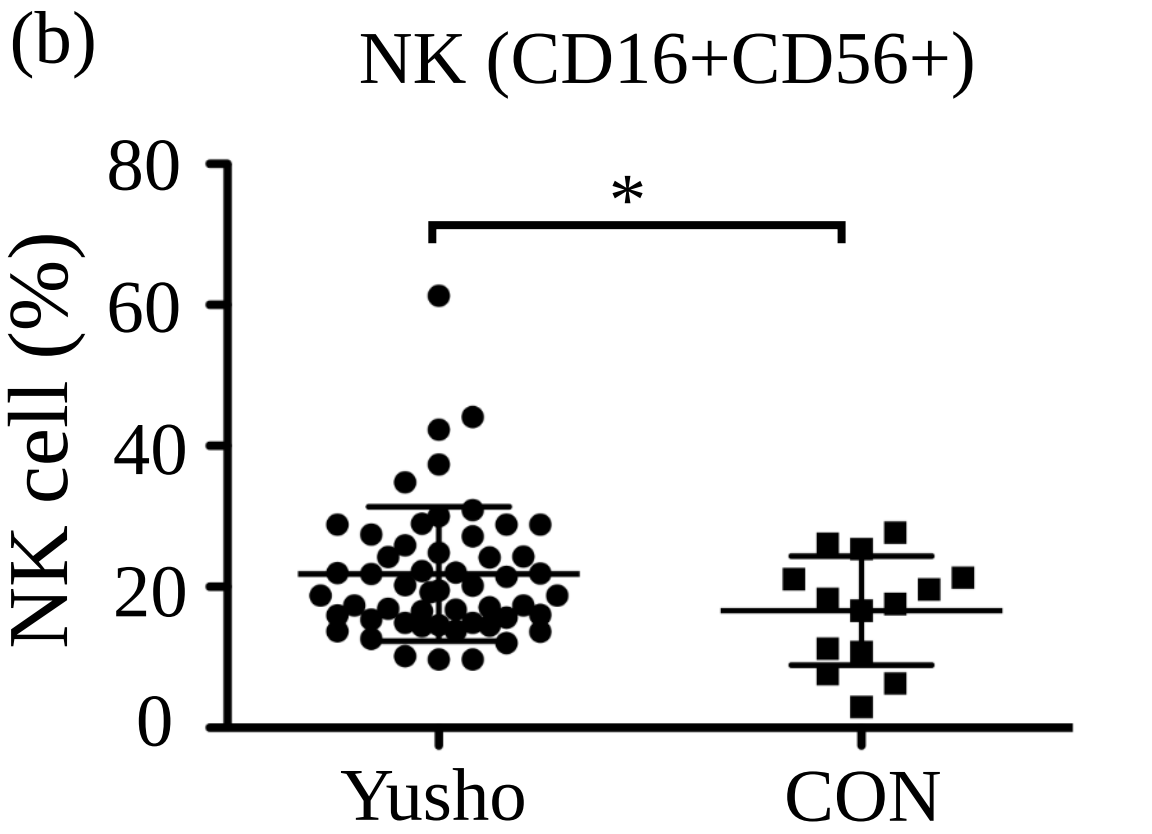

(b)
NK (CD16+CD56+)
*
NK cell (%)
80
60
40
20
0
Yusho
CON

Figure.2

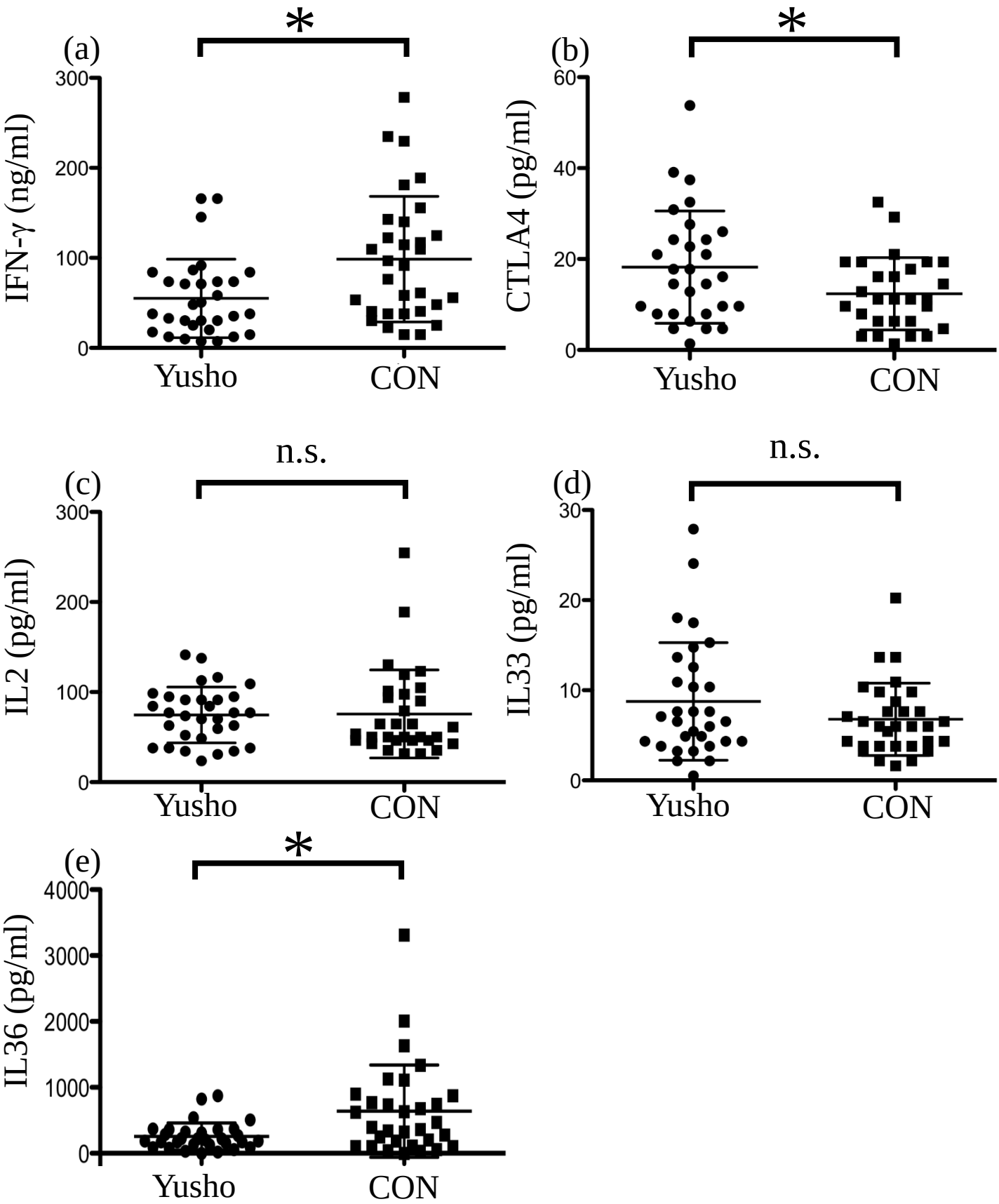
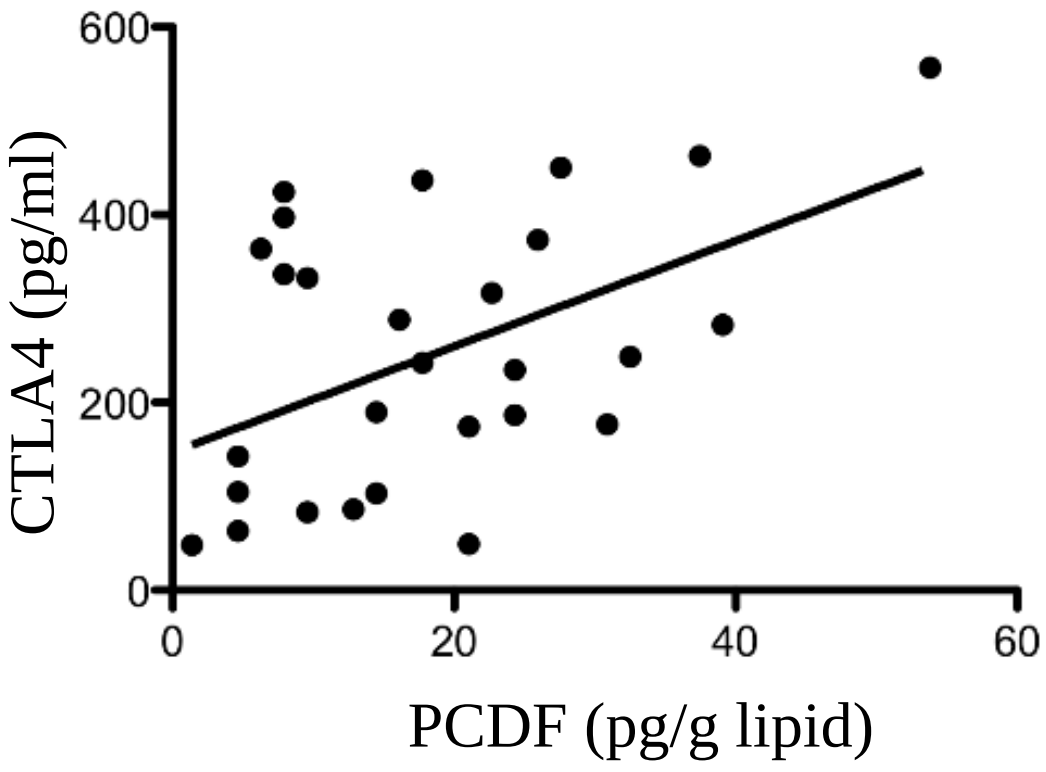

(a)
*
IFN-γ (ng/ml)
300
200
100
0
Yusho
CON
(b)
*
CTLA4 (pg/ml)
60
40
20
0
Yusho
CON
(c)
n.s.
IL2 (pg/ml)
300
200
100
0
Yusho
CON
(d)
n.s.
IL33 (pg/ml)
30
20
10
0
Yusho
CON
(e)
*
IL36 (pg/ml)
4000
3000
2000
1000
0
Yusho
CON

Figure.3

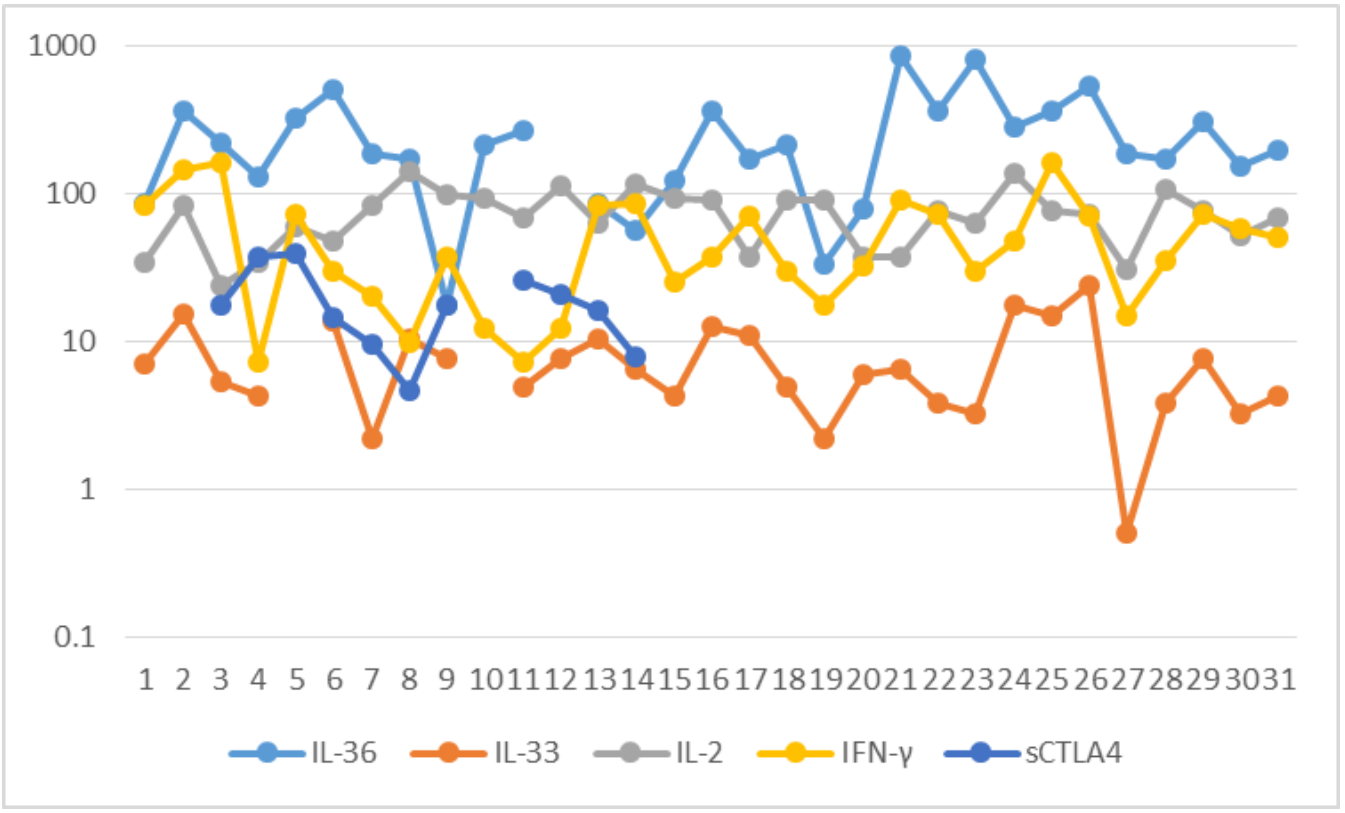
(a)

	PCDF (pg/g lipid) n=24	**PCQ (ppb) n=23**	**PCB (ppb) n=24**
IFN-γ (ng/ml)	*p*=0.3944 *r*=0.1821	*p*=0.3192 *r*=0.2173	*p*=0.8319 *r*=0.04574
CTLA4 (pg/ml)	* ***p*=0.0302** ***r*=0.4030**	*p*=0.3741 *r*=-0.1714	*p*=0.9195 *r*=0.01962
IL2 (pg/ml)	*p*=0.7170 *r*=-0.07803	*p*=0.8769 *r*=-0.03418	*p*=0.7170 *r*=-0.07803
IL33 (pg/ml)	*p*=0.5430 *r*=0.1306	*p*=0.3568 *r*=-0.2014	*p*=0.2571 *r*=-0.2407
IL36 (pg/ml)	*p*=0.3779 *r*=-0.1884	*p*=0.5362 *r*=0.1360	*p*=0.6934 *r*=0.08486

(b)



Yusho



Control