Dual effects of interleukin-10 on natural killer cells and monocytes and the implications for adult-onset Still's disease

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ABSTRACT

Objective. *We compared the serum levels of multiple cytokines in patients with adult-onset Still's disease (AOSD) and healthy controls to assess the effects of humoral factors on natural killer (NK) cells and monocytes.*

Methods. *We quantified the serum levels of 10 cytokines in the patients using bead-based multiplex immunoassays, along with interleukin (IL-)18 using ELISA. We then sorted NK cells and monocytes from the peripheral blood mononuclear cells (PBMCs) of healthy volunteers, cultured them in the presence or absence of cytokines that were detected in some or all of the serum samples from the AOSD patients and their combinations in vitro, and analysed the culture supernatant.*

Results. *IL-6 and IL-18 were the main cytokines increased in the serum of AOSD patients. When NK cells were cultured with the cytokines and IL-10, the combination of IL-10 and IL-18 substantially induced interferon (IFN-)γ. IL-6 had little effect on NK cells, probably because they barely expressed the IL-6 receptor and glycoprotein 130 (gp130). IFN-γ induced monocytes to produce IL-1β, IL-6 and tumour necrosis factor (TNF-)α whereas IL-10 inhibited the induction of these proinflammatory cytokines.*

Conclusion. *IL-10 evidently has dual effects on NK cells (stimulation) and on monocytes (inhibition). Better understanding the roles of the cytokine network would shed light on the pathogenesis of AOSD.*

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Introduction

Adult-onset Still's disease (AOSD) is a systemic inflammatory disease, the causes of which remain largely unknown. AOSD is characterised by a daily spiking fever, arthritis and rash (1). In addition to the inflammatory marker C-reactive protein (CRP), serum ferritin is also considered to be an activity marker of the disease. Recently, serum aldolase was reported to be a useful diagnostic and activity marker (2). It has been considered closely related to collagen vascular diseases, but specific autoantibodies have not been established. Thus, B cells that produce antibodies and T cells that help antibody production may not be much involved in its pathogenesis; in other words, acquired immunity may not play a primary role. This is one of the reasons why it has recently been reclassified as an "autoinflammatory disease", not as an autoimmune disease (3). Autoinflammatory diseases are a group of diseases in which innate rather than acquired immunity plays the critical role in the pathogenesis (4). Monocytes/macrophages, granulocytes, natural killer (NK) cells and γ/δ T cells are among the immune cells that play important roles in innate immunity. They can stimulate each other by producing various proinflammatory cytokines, which is probably the cause of the persistent inflammation that is the hallmark of the condition. AOSD is known for a high serum concentration of interleukin (IL-)18 (5, 6), which was originally identified as a cytokine that induces the production of interferon (IFN-)γ (7).

We and other groups have reported that the anti-IL-6 receptor antibody tocilizumab is effective against AOSD (8-10), indicating that IL-6 plays an important role in the pathogenesis of AOSD. This is consistent with the fact that the level of CRP is usually very high in untreated AOSD patients.

In this study, we first attempted to quantify the levels of multiple cytokines in the serum of AOSD patients by utilising the Bio-Plex Multiplex Immunoassay, a bead-based technique. We then focused on the effects of the cytokines detected in the sera of AOSD patients on the NK cells and monocytes derived from healthy controls.

Materials and methods

Patients

Peripheral blood was obtained from 16 AOSD patients who were admitted to Saitama Medical University Hospital or were visiting the outpatient clinic and 8 healthy controls. Most of the patients were symptomatic at the time of sampling. AOSD was diagnosed according to the Yamaguchi criteria of 1992 (11). This study was approved by the Institutional Review Board of Saitama Medical University Hospital, Japan (No. 13085) and conformed to the provisions of the World Medical Association's Declaration of Helsinki. Written informed consent was obtained from each patient.

Bead array assay and ELISA

The serum levels of the multiple cytokines were quantified with the Bio-Plex Pro Assay using a Bio-Plex 200 system (Bio-Rad) according to the manufacturer's protocol (12). The cytokines quantified were: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A and tumour necrosis factor (TNF-) α. The serum IL-18 level was measured with a Human IL-18 ELISA Kit (MBL) according to the manufacturer's instruction. The level of IFN-γ in the culture supernatant was quantified with a Human IFN-γ Quantikine ELISA Kit (R&D) and the levels of IL-1β, IL-6 and TNF-α in the culture supernatant were quantified with the BD Cytometric Bead Array (BD Biosciences) according to the manufacturers' protocols.

The values below the lowest standard curve points (the lower limit of quantification, ULOQ) were treated as equal to ULOQ.

Cell isolation and culture

Mononuclear cells were isolated from peripheral blood by Ficoll gradient centrifugation. NK cells were then sorted using autoMACS and the Human NK Cell Isolation Kit (Miltenyi Biotec) (13, 14). Monocytes were sorted using an EasySep Magnet and "EasySep Human Monocyte Enrichment Kit without CD16 Depletion" reagent (STEMCELL Technologies) (15, 16). The sorted cells were cultured in RPMI1640 medium containing 10% FBS (2 x 10^6/mL) for 15 h in the presence or absence of 10 ng/mL IL-6, IL-10, IL-18 and/or IFN-γ (IL-18 was purchased from R&D systems. The other cytokines were from Peprotech). Subsequently, cells were stained with anti-IL-6R (CD126), anti-gp130 (CD130), anti-IL-10Rα (CD210) or anti-IL-10Rβ (CDw210b) antibodies

Table I. Demographic of AOSD patients and healthy controls (HC).

	AOSD	HC
Number	16	8
Age	49.9 ± 16.0	35.6 ± 12.6
Gender (male/female)	8/8	5/3
CRP (mg/dL)	12.5 ± 3.99	NA
Prednisolone (mg/day)	6.0 ± 6.5	NA
Ferritin (mg/dL)	299 ± 360	NA

NA: not available.

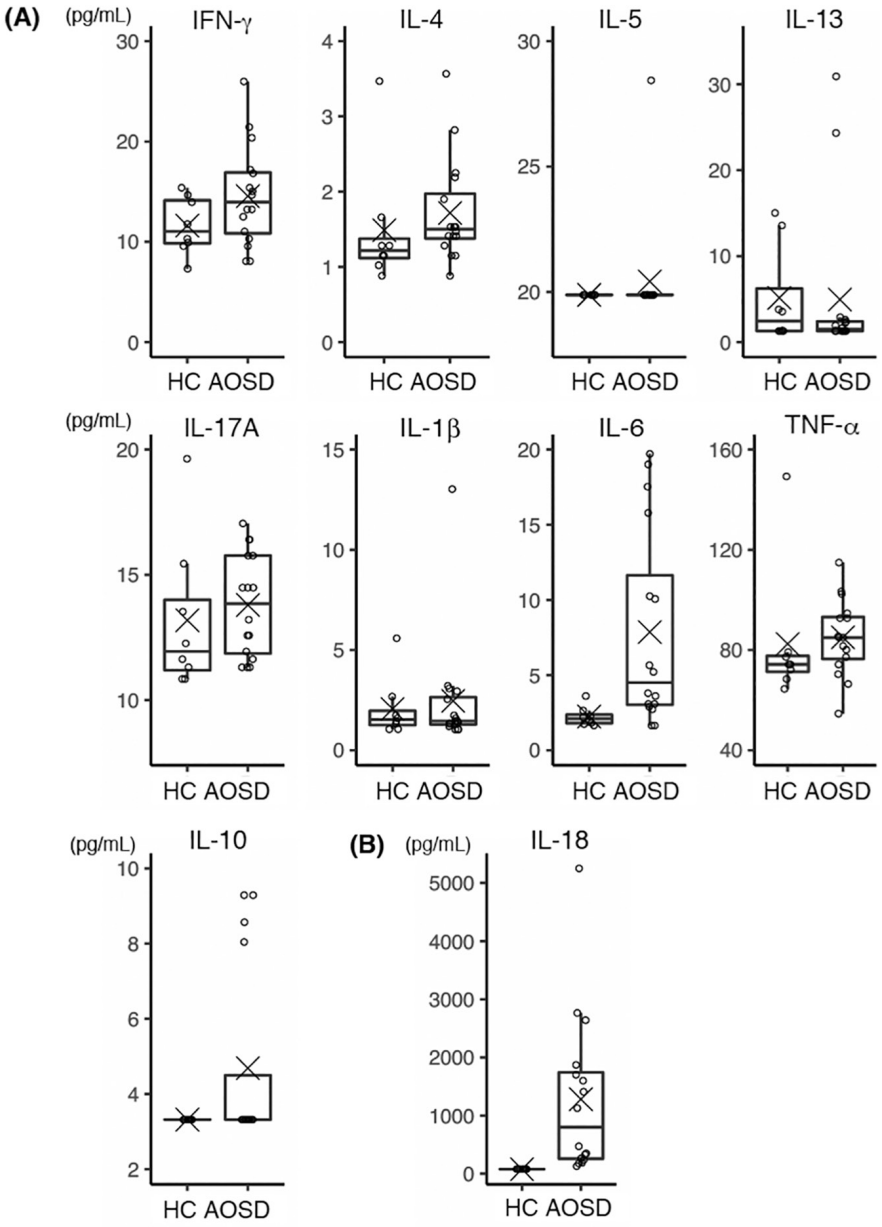

Fig. 1. Box plots overlaid with dot plots of the levels of various cytokines in the serum of healthy controls and AOSD patients quantified with the Bio-Plex Multiplex Immunoassay (**A**) and ELISA (**B**). Cross marks indicate average values.

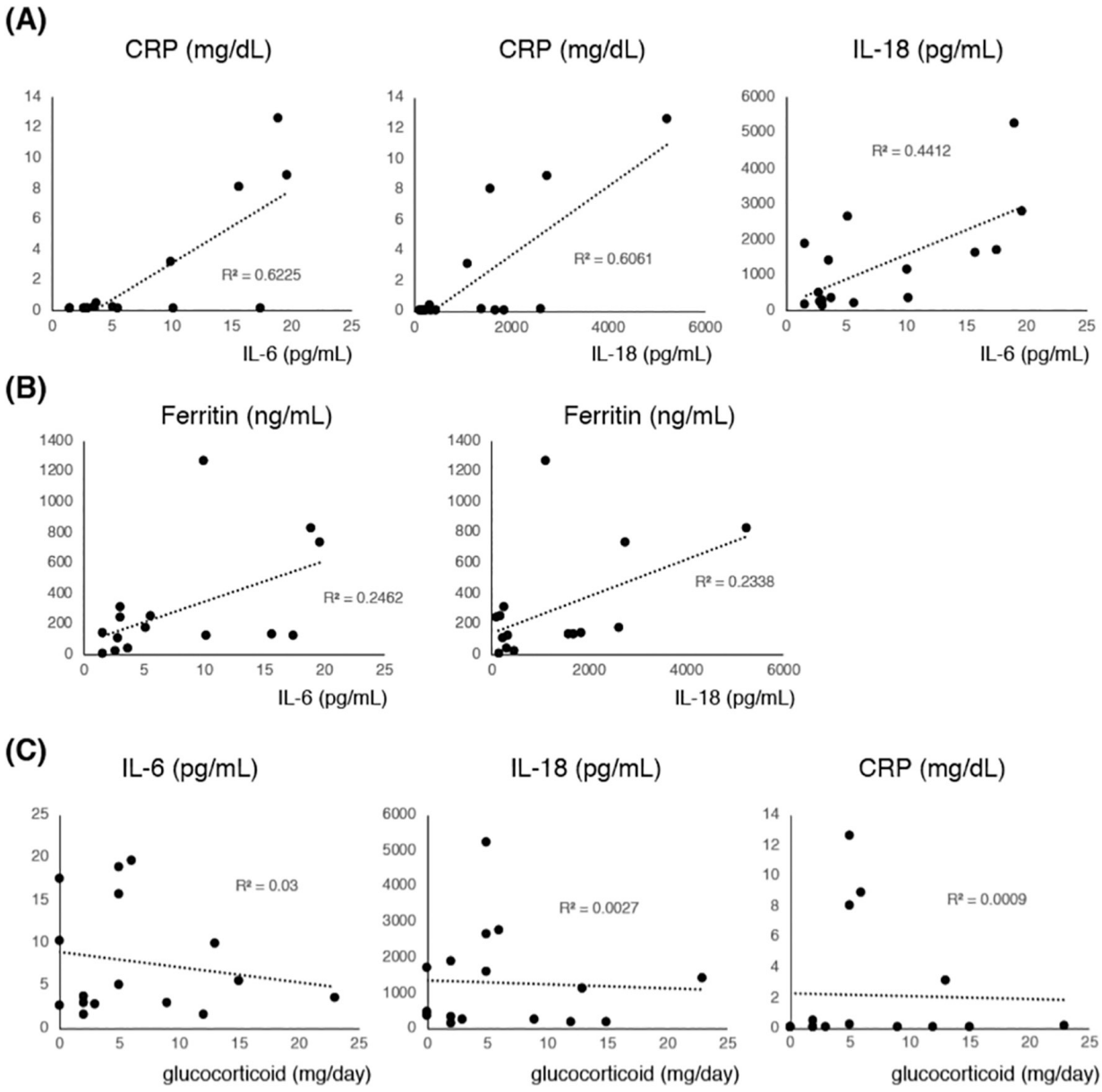

Fig. 2. Scatter plots with linear regression lines of the cytokine levels and clinical parameters.
A: Correlation among the serum levels of CRP, IL-6 and IL-18. **B**: Correlation between the serum levels of ferritin and IL-6 or IL-18.
C: Relationship between the dosage of glucocorticoid (prednisolone equivalent) and the serum levels of IL-6, IL-18 and CRP.

conjugated with phycoerythrin in the presence of FcR Blocking Reagent. The former three antibodies were from BioLegend and the latter two reagents were purchased from Miltenyi Biotec. Anti-CD56 antibodies conjugated with allophycocyanin (APC) and anti-CD3 antibodies conjugated with fluorescein isothiocyanate (both from BioLegend) were used for the gating of NK cells. Cells were stained with anti-CD16-APC antibodies and anti-CD14-APC-H7 antibodies (both from BD Biosciences) for monocyte gating. Dead cells were excluded from the analysis by 7-Amino-Actinomycin D labelling (Miltenyi Biotec). The cells were then analysed using a FACSCanto II (BD Bioscience) (17). FlowJo software (TreeStar) was used for data analysis.

Statistical analysis

Statistical significance was tested using the Mann-Whitney U-test. A p-value of <0.05 was considered as statistically significant.

Results

First, we quantified the levels of 10 cytokines in the serum sample derived from the AOSD patients and healthy controls with the Bio-Plex Multiplex Immunoassay (Fig. 1A). In addition, the level of IL-18, which cannot be assayed simultaneously in the assay system, was quantified with ELISA (Fig. 1B). The levels of IFN-γ, a representative cytokine of the Th1-type response, were not substantially different between the AOSD patients and healthy controls. IL-2, another so-called Th1 cytokine, was barely detected in the two groups (data not shown). Th2 cytokines, namely, IL-4, IL-5 and IL-13, and the canonical Th17 cytokine IL-17

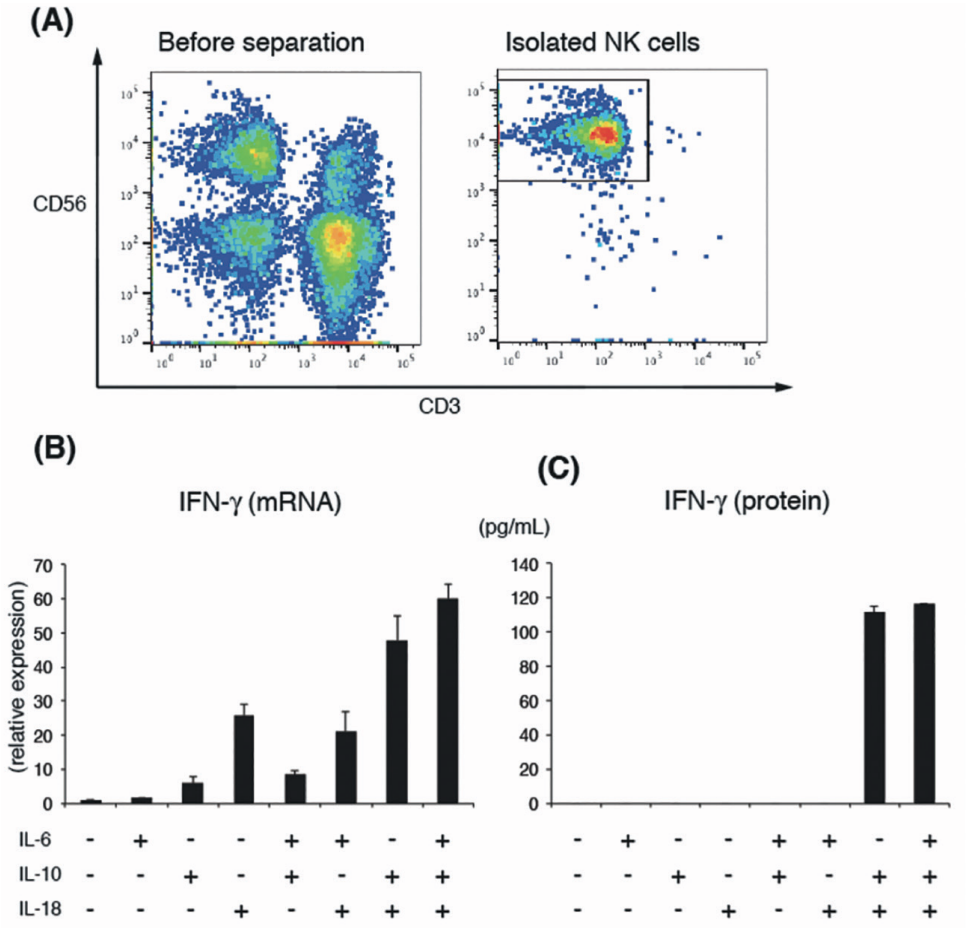

Fig. 3. **A**: Human NK cells were sorted from the PBMCs of healthy controls.
B: IFN-γ expression at the mRNA level (mean ± standard deviation) in the NK cells stimulated with various combinations of IL-6, IL-10 and IL-18.
C: IFN-γ expression at the protein level in the culture supernatants of the NK cells. Data are representative of three independent experiments.

(IL-17A) were not very different, either. Among the classical proinflammatory cytokines IL-1β, IL-6 and TNF-α, only IL-6 was significantly higher in the AOSD patients (p=0.0064). The levels of IL-10, which is supposed to be an anti-inflammatory cytokine, were substantially higher in the AOSD patients, although only 4 out of 16 samples were above the detection limit. As expected, significantly higher levels of IL-18 were detected in the serum of AOSD patients than controls (p=6.5 x 10^{-5}, Fig. 1B).

The levels of serum IL-6 and CRP were highly correlated, which is to be expected, since IL-6 is important for the induction of CRP (18). A similar level of correlation was observed between serum IL-18 and CRP. This was also expected since there was a substantial correlation between serum IL-6 and IL-18 (Fig. 2A). On the other hand, a weaker level of correlation was observed between IL-6 or IL-18 and AOSD activity marker ferritin (Fig. 2B). The dosage of glucocorticoid (prednisolone equivalent) and serum IL-6 or IL-18 were weakly correlated (Fig. 2C). It also did not correlate with CRP.

Since NK cells are involved in innate immunity and AOSD has been recently reclassified as an autoinflammatory disease (3), they are candidate cells for the production of IFN-γ in response to IL-18. Thus, we sorted NK cells from the peripheral blood mononuclear cells of healthy controls (Fig. 3A) and cultured them *in vitro* in the presence of IL-6, IL-10, IL-18 or a combination of these cytokines. When IFN-γ was quantified at the mRNA level using qRT-PCR, IL-10 and IL-18 induced the cytokine, but IL-6 only barely induced it. The combination of IL-10 and IL-18 induced it most (Fig. 3B). At the protein level, this tendency became more pronounced; the IFN-γ protein was only detected in the supernatant samples of the cells cultured with IL-10 and IL-18 (Fig. 3C). In contrast, IL-6 did not have any evident effect on IFN-γ.

IL-18 reportedly induces TNF-related apoptosis-inducing ligand (TRAIL) in liver NK cells in an IFN-γ-dependent manner (19). Thus, we assessed TRAIL expression on NK cells. In this case, too, the combination of IL-10 and IL-18 induced the expression of TRAIL on NK cells the most (Fig. 4A, left panels). The histogram of the mean fluorescence intensity (MFI) was strikingly similar to that of the mRNA level of IFN-γ (Fig. 4B, left panel and 3B). The same combination barely induced the expression of FASL (Fig. 4A, right panels). This induction of TRAIL by IL-10 and IL-18 was not inhibited by anti-IFN-γ mAbs (Fig. 4C).

It was unexpected that IL-6 had so little an effect on the expression of IFN-γ and TRAIL on NK cells. Thus, we analysed the expression of IL-6 receptors and IL-10 receptors on NK cells and found that, although IL-10Rα and IL-10Rβ were both expressed, IL-6R and gp130 were barely expressed (Fig. 4D). IFN-γ is known to stimulate monocytes (20, 21). Monocytes derived from peripheral blood expressed not only IL-10 receptors but also IL-6 re-

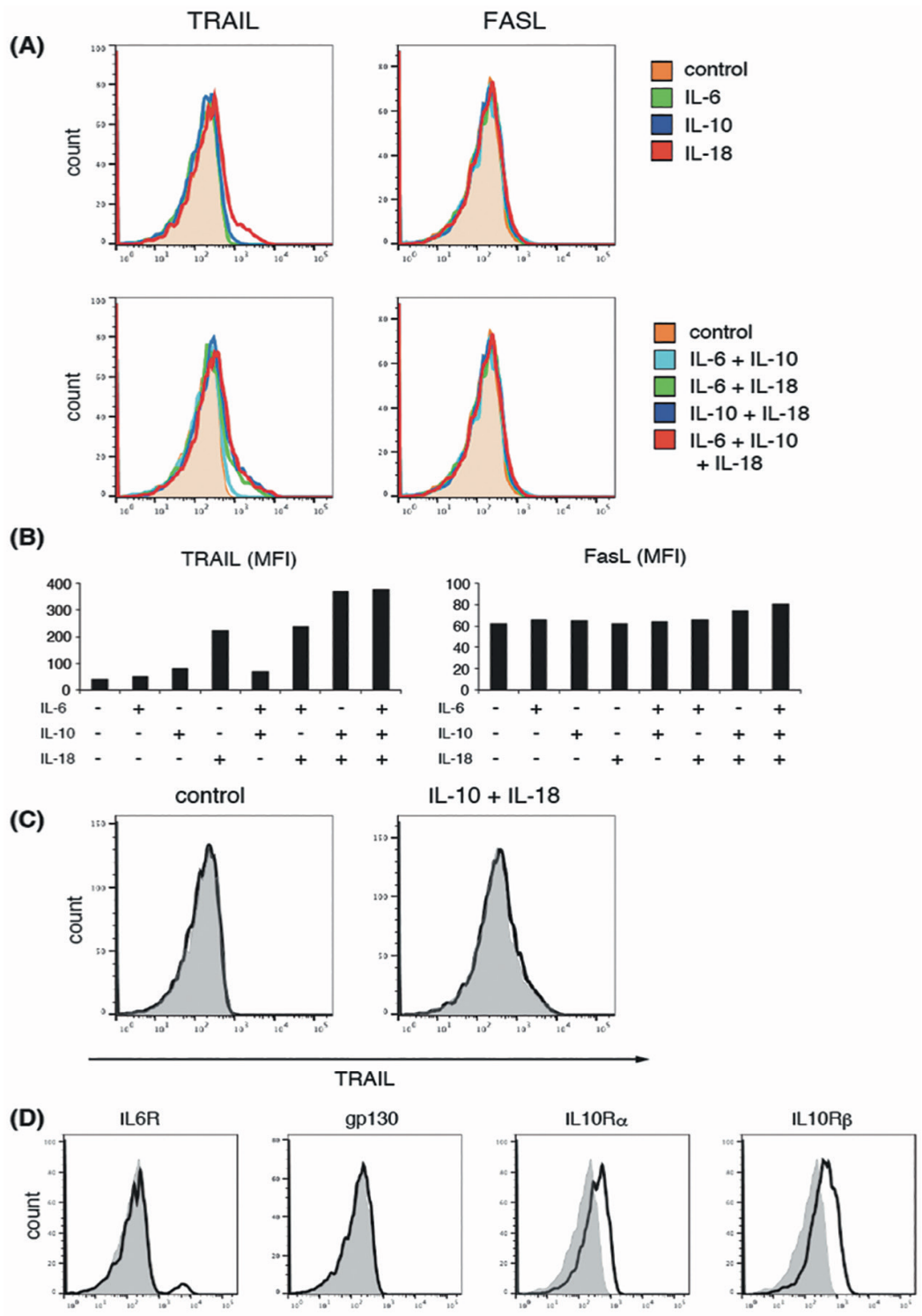

Fig. 4. **A**: Histograms of the surface expression of TRAIL and FASL on NK cells.
B: Histograms of mean fluorescence intensity (MFI) of the two ligands on NK cells.
C: Effect of anti-IFN-γ antibodies on the expression of TRAIL. The shaded areas represent the cells cultured in the presence of control IgG.
D: Surface expression of IL-6 and IL-10 receptors on NK cells. Data are representative of three independent experiments.

ceptors (Fig. 5A). In order to assess the effect of IFN-γ, IL-6, IL-10 and their combination on monocytes, we cultured monocytes in the presence or absence of these cytokines and quantified the levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α in the culture supernatant. As expected, IFN-γ enhanced the levels of all three proinflammatory cytokines. IL-10 did not enhance them but rather, clearly inhibited the production of them (Fig. 5B).

Discussion

In this study, we first quantified 11 cytokines in the serum of AOSD patients. Consistent with former studies, IL-18 was above the detection limit in all of the serum samples derived from the AOSD patients and was significantly higher than in healthy controls. Fourteen out of 16 of the samples were IL-6 positive, which is also not surprising in that IL-6 is one of the representative inflammatory cytokines and that anti-IL-6R mAbs have been shown to

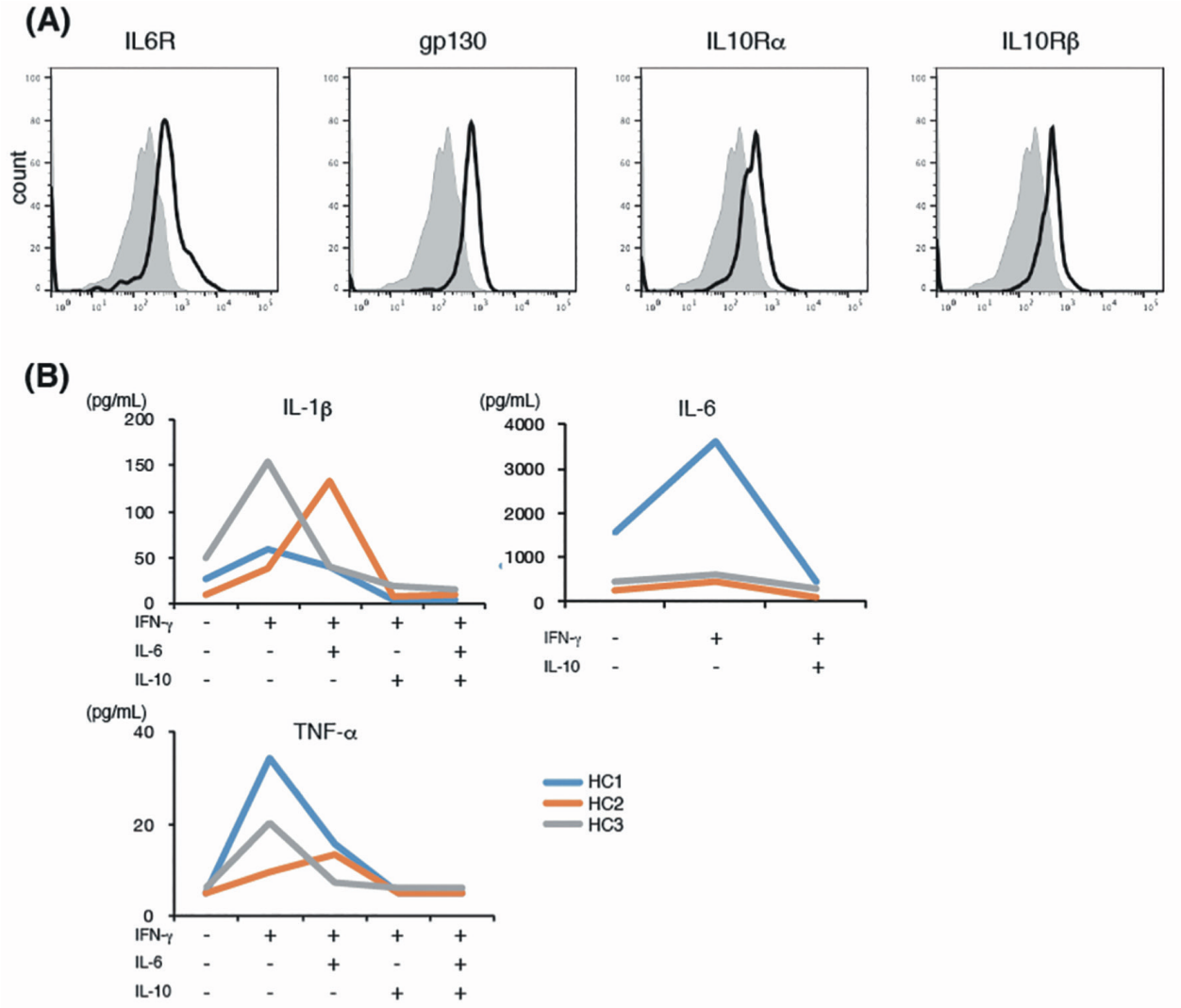

Fig. 5. A: Surface expression of IL-6 and IL-10 receptors on CD14 and/or CD16 positive monocytes. **B**: Amount of proinflammatory cytokines in the culture supernatants of monocytes stimulated with various combinations of IFN-γ, IL-6 and IL-10. Data are a combination of three independent experiments.

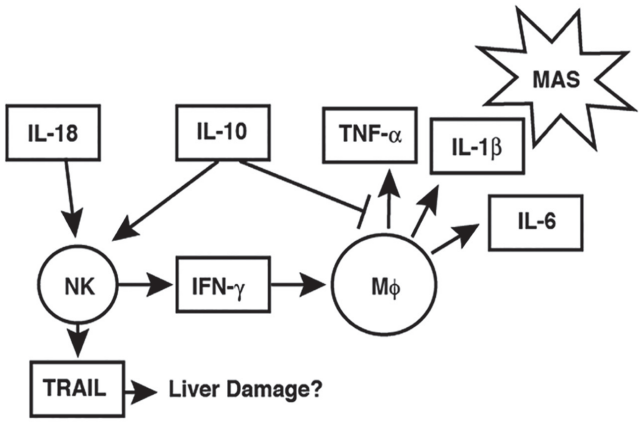

Fig. 6. Summary figure of the interaction between NK cells and monocytes that occurs via various cytokines.

be effective against AOSD (9, 10). IL-6 is also indispensable for the production of CRP (18), and AOSD is a disease characterised by a high level of serum CRP (1).

The biological activity of IL-18 is regulated by IL-18 binding protein (IL-18BP), which binds IL-18 with a high affinity, and thereby prevents the interaction of IL-18 with its receptor (22). In this study, we quantified the total IL-18 level. Jung *et al*. quantified both the total IL-18 and IL-18BP in AOSD patients and controls and calculated the free IL-18 level based on the law of mass action (23). They found that there was a strong positive correlation between total IL-18 and free IL-18 (r = 0.992). Taking both time and cost into consideration, the authors suggested that total IL-18 is an appropriate marker in AOSD patients. Girard *et al*. developed a free IL-18 specific ELISA and directly quantified the serum levels of free IL-18 in AOSD patients (24). They, too, demonstrated that free IL-18 and total IL-18 were highly correlated (r = 0.9335). Thus, we can accept that the significantly higher level of total IL-18 in AOSD patients does reflect

the biological activity of IL-18 in this study.

The level of serum IFN-γ was not substantially different between the AOSD patients and healthy controls, which is a little surprising since IL-18 was originally identified as a factor that stimulates IFN-γ production (7). Thus, we assumed that IFN-γ might be produced locally but not sistemically in AOSD patients. Since IFN-γ is a strong stimulator of monocytes (20, 21), if it were produced systemically, monocytes throughout the body may be activated. This would cause macrophage activation syndrome (MAS), which can be extremely dangerous to the body as a whole. Indeed, AOSD is a disease in which MAS can become a problem (1, 25). NK cells are among candidate cell types for producing IFN-γ in response to IL-18. They are an especially attractive target to investigate since NK cells are classified as cells belonging to innate immunity and AOSD is now considered as an autoinflammatory disease (3).

In addition to IL-6 and IL-18, we chose IL-10 as a cytokine to be added to the culture of NK cells *in vitro*, although IL-10 was detected in only a quarter of the serum samples analysed. In fact, IL-10 has been reported to be present in the serum of AOSD patients (26, 27). Sun *et al*. reported that the serum levels of IL-10 in AOSD patients were positively correlated with disease activity (26, 27). As IL-10 has been considered a typical anti-inflammatory cytokine, the authors interpreted the expression as a compensatory mechanism against inflammation. Since the serum concentration of IL-10 was rather low, their assumption was that the expression of IL-10 was insufficient to control the inflammation in AOSD (28). Yet another possibility is that the expression of local IL-10 was more pronounced than that in the serum. This is the background of why we added IL-10 to the NK-cell culture. Interestingly, NK cells did not produce IFN-γ at the protein level when stimulated with IL-18 alone. In the presence of IL-18 and IL-10, however, they substantially produced the cytokine. These results apparently contradict the notion that IL-10 is an anti-inflammatory cytokine in all contexts.

Tu *et al*. reported that IL-18, in combination of TLR3 signalling, induced TRAIL on NK cells in an IFN-γ dependent manner (19). Indeed, IL-18 stimulation induced TRAIL on NK cells. Thus, it seemed likely that IL-18 induced TRAIL expression via IFN-γ. The addition of anti-IFN-γ mAbs, however, did not inhibit the induction of TRAIL (Fig. 4C). Moreover, in the paper by Tu *et al*., IL-10 diminished TRAIL expression, in contrast with our results. Thus, the induction of TRAIL on NK cells by the combination of IL-18 and IL-10 is likely to be due to IFN-γ -independent mechanisms. In fact, IL-10 is reported to upregulate TRAIL in mammary epithelial cells (29).

What is the significance of this TRAIL induction? TRAIL belongs to the TNF family of cytokines. Because of its tumoricidal activity, TRAIL or an agonistic reagent against its receptor had been expected to be useful as novel drugs for anti-cancer therapy (30, 31). However, hepatotoxicity can become a problem, as in the case with FASL (32, 33). Abnormal liver function tests are among the diagnostic criteria for AOSD (11). TRAIL on NK cells may be involved in such liver dysfunction.

The fact that NK cells did not respond to IL-6 was also a bit surprising. As it turned out, NK cells do not express IL-6 receptors. Even if NK cells lack membrane-bound IL-6R, they may be able to respond to IL-6 because soluble IL-6R substitutes for the membrane bound form. Since they also lack gp130, however, it is unlikely that they respond to IL-6. Recently, granulocytes were reported to be unresponsive to IL-6 due to the absence of gp130 (34). Thus, the target cells of IL-6 in the pathogenesis of AOSD might not be NK cells or granulocytes.

We finally analysed the effect of IL-10 on monocytes stimulated with IFN-γ. As expected, IFN-γ induced the production of proinflammatory cytokines IL-1β, IL-6 and TNF-α from monocytes, and IL-10 clearly inhibited this induction. The combination of IL-10 and IL-18 did not induce proinflammatory cytokines from monocytes (data not shown). Thus, in this case IL-10 apparently acted as a negative regulator of inflammation.

The limitation of this study is that the analyses of the NK cells and monocytes were performed using cells derived from the PBMCs of healthy controls. Indeed, NK cells from the PBMCs of AOSD patients reportedly have weaker cytotoxic activity (35), suggesting that they are in a state of exhaustion. As NK cells are known to be rich in the liver, it would be ideal to analyse NK cells of the liver from AOSD patients, if possible. The same applies to monocytes. Hepatic macrophages consist of not only infiltrated bone marrow-derived monocytes/macrophages but also of Kupffer cells, which originate from the foetal yolk-sack (35). Such macrophages may be responsible for the liver dysfunction observed in AOSD. Despite the difficulty of analysing such cells from AOSD patients, it will be an important task to do so in the future.

It must also be noted that the disease activity of AOSD is highly affected by the treatment itself, including glucocorticoids as well as other immunosuppressants. In this study, some patients needed intensification of their treatment after the blood sample had been obtained, whereas other patients exhibited a low level of disease activity even on only a low dosage of glucocorticoids. Thus, the specific relationship between the degree of therapy and the markers of disease activity is difficult to evaluate, as shown in Fig. 2C. A time-course analysis would be necessary to actually determine the relationship between the treatment and the activity markers, and that was beyond the scope of this investigation.

In summary, the combination of IL-18 and IL-10, which can be detected in the serum of AOSD patients, induces IFN-γ production from and TRAIL expression on NK cells *in vitro*. Subsequently, IFN-γ induces proinflammatory cytokines IL-1β, IL-6 and TNF-α from monocytes. Contrastingly, IL-10 downregulates the induction of these proinflammatory cytokines (Fig. 6). Thus, IL-10 evidently has dual effects on NK cells and monocytes, a feature which may play an important role in the pathogenesis of AOSD.

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