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# Reactive oxygen species in hematopoietic stem cells affect culture outcomes under inflammatory conditions

*Takashi Ishida[1,2], Satoshi Yamazaki[2], Hiromitsu Nakauchi[2], Masaaki Higashihara[1], and Makoto Otsu[3,*]*

[1] Department of Hematology, Kitasato University Graduate School of Medical Science, Kanagawa, Japan

[2] Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan

[3] Division of Stem Cell Processing and Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan

**Corresponding Author & Address:**

*Makoto Otsu*[*]

Division of Stem Cell Processing and Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, 108-8639 Tokyo, Japan; Tel: +81-3-6409-2342; Fax: +81-3-6409-2343; Email: motsu@ims.u-tokyo.ac.jp

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## ABSTRACT

*Functions of hematopoietic stem cells (HSCs) are tightly regulated to ensure lifelong normal hematopoiesis within the bone marrow (BM) niche. While HSCs' status is well orchestrated to uphold their stemness, the fate of HSCs can be altered in response to inflammatory signals caused by environmental changes. The influence on HSCs of inflammatory changes within BM has been explored; however, how each inflammatory cytokine affects the maintenance or differentiation of HSCs remains to be fully elucidated. Considering recent studies demonstrating that reactive oxygen species (ROS) influence HSCs' fates, we conducted a comprehensive analysis of the effects of seven major inflammatory cytokines on proliferation, differentiation, and ROS level regulation in HSCs / hematopoietic stem/progenitor cells (HSPCs). Our study indicates that inflammatory cytokines in general push HSCs/HSPCs into differentiation, but vary significantly with respect to retention of primitive cells. With definition by cellular immunophenotypes, TNF-α, IFN-γ, and type I IFNs stimulated loss of HSCs, whereas TGF-β and IL-6, and possibly IL-1β as well, showed potential to retain them. Of note is that unaltered ROS levels were compatible with HSC retention, while drastic change, either increase or decrease, in ROS levels presaged HSC loss. Detailed evaluation of links between inflammatory cytokines and ROS regulation appears likely to prove important in understanding HSC biology and translating basic science into clinical applications.*

## INTRODUCTION

Hematopoietic stem cells (HSCs) are characterized by their potential for self-renewal and multi-lineage differentiation, making them able to maintain lifelong hematopoiesis. HSCs reside within the bone marrow (BM) niche. They are tightly regulated by extrinsic cues to sustain hematopoietic homeostasis [1]. HSCs in the steady state are quiescent [2], but their fate can be altered in response to environmental changes.

Recent studies have reported that inflammation may be one of the major stresses on HSCs, affecting their lineage commitment, self-

renewal, homing ability, and susceptibility to apoptosis [3-6]. In some blood disorders such as hematopoietic malignancies, BM failure is reported to be linked with the overproduction of inflammatory cytokines [3, 7]. Although one can speculate that HSCs may be regulated to exhibit compensatory responses to certain stresses [8-10], it remains unclear how drastic environmental changes can lead to dysregulation of HSCs' functions and thereby to hematopoietic abnormalities. Therefore, detailed analysis of inflammatory cytokines' effects on the functionality of HSCs is desirable.

We here sought to determine the effects of various inflammatory cytokines on cultured hematopoietic stem/progenitor cells (HSCs/HSPCs) by an in vitro comprehensive analysis using human CD34+ cells. We selected 7 candidate cytokines: Interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interferon-alpha (IFN-α), interferon-beta (IFN-β), interferon-gamma (IFN-γ), and transforming growth factor-beta (TGF-β). We investigated how treatment with each individual cytokine affected cell proliferation and differentiation in the presence of a baseline cytokine cocktail comprising stem cell factor (SCF), thrombopoietin (TPO), and Flt3-ligand (FL). We also tested how these inflammatory cytokines influenced cellular levels of reactive oxygen species (ROS), which have been shown to affect the fate of HSCs [11].

## MATERIALS AND METHODOLOGY

### Isolation of human CD34+ cells

Umbilical cord blood (UCB) units obtained at birth were kindly provided by the Japanese Red Cross Kanto-Koshinetsu Block Blood Center. To isolate mononuclear cells (MNCs), UCB units were subjected to density-gradient separation (Ficoll; Immuno-Biological Laboratories, Gunma, Japan). CD34+ cells were then isolated from MNCs using a CD34 Microbead Kit (Miltenyi Biotech, Bergisch Gladbach, Germany).

### Cell Culture

CD34+ cells were aliquoted at 5 x 104 cells into individual wells of 96-well flat-bottom plates containing X-VIVO10 (Lonza, Basel, Switzerland) supplemented with 1% human serum albumin (CSL Behring, King of Prussia, PA), 1% penicillin-streptomycin-glutamine (Life Technologies, Carlsbad, CA), and 50 ng/ml human SCF, TPO, and FL (Peprotech, Rocky Hill, NJ). Cells were cultured at 37oC in 5% CO2 for 2 days or, with the addition of fresh medium on day 3, for 5 days. In some experiments, culture medium contained, for the entire period one of these inflammatory cytokines: IL-1β (10 ng/ml), TGF-β, TNF-α, IFN-γ, IFN-β, and IL-6 (all 50 ng/ml; all Peprotech), and universal type I IFN-α (1,000 U/ml; PBL Assay Science, Piscataway, NJ). After culture, cells were harvested for downstream assays, including cell count determinations using a CYTOREGON automatic cell image counter (GE Health Science, Little Chalfont, UK).

### ROS detection assay

Cells collected after 2 days of culture were stained with HySOx, a newly developed ROS indicator (excitation: 555 nm, emission: 575 nm; Goryo Chemical, Hokkaido, Japan), for 30 minutes at 37oC. After washing, cells were analyzed for ROS accumulation by FACS Aria II flow cytometry (BD Biosciences, San Jose, CA). Cellular ROS were quantified by mean fluorescence intensity (MFI).

### Flow cytometry analysis of cell surface markers

Cultured CD34+ cells were stained with the following monoclonal antibodies: phycoerythrin (PE)-cyanine 7-conjugated anti-CD38 (eBioscience, San Diego, CA), allophycocyanin (APC)-cyanine 7-conjugated anti-CD45, Pacific Blue-conjugated anti-CD34, and the proprietary reagent fluorescein isothiocyanate (FITC) anti-human Lineage Cocktail (Biolegend, San Diego). After washing, cells were subjected to flow cytometry (FACS Aria II or Canto, BD Biosciences). All data were analyzed using FlowJo software (TreeStar, Ashland, OR).

### Statistical Analysis

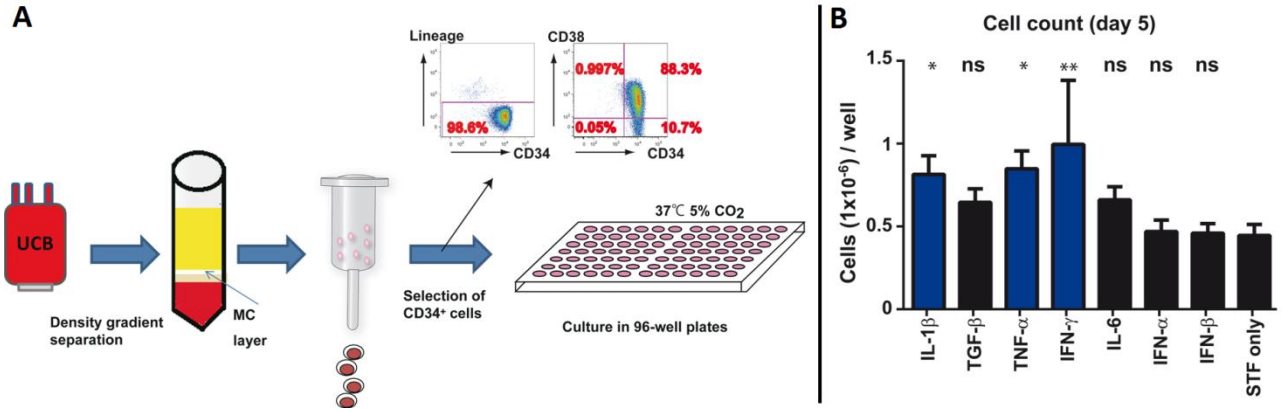
One-Way-ANOVA testing was used for statistical analysis. The value of each group was compared with that of a control group using Dunnett's post hoc test. A p value $< 0.05$ was considered statistically significant. The number of asterisks corresponds to the degree of P value, with *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; and ****: $P < 0.0001$.

## RESULTS AND OBSERVATIONS

To examine the effects of inflammatory

cytokines on HSCs/HSPCs, we used highly purified CD34+ cells obtained from human UCB. As shown in Figure 1A, CD34+ cell purity was routinely > 95%, with retention at a frequency of ~10% of a more primitive cell population with CD34+/CD38- phenotype. These cells were cultured in serum-free medium supplemented with a baseline cytokine cocktail containing SCF, TPO, and FL. Their proliferative response was assessed 5 days after initiation of culture (Figure 1A). As shown in Figure 1B, the stated conditions (STF only) expanded cell numbers (50,000 input cells) by ~10 times. Addition of each inflammatory cytokine variously affected overall proliferation of HSCs/HSPCs. Two type I interferons (IFN-α and IFN-β) effected virtually no change in cell numbers versus STF only. Other cytokines, however, in general increased numbers of cultured HSCs/HSPCs. Cell number expansion by IL-1β, TNF-α, and IFN-γ reached statistical significance.

**Figure 1:** Effects of inflammatory cytokines on proliferation of HSCs / HSPCs.




(A) Schematic representation of experiments to test effects of inflammatory cytokines on human CD34-positive (CD34+) cells derived from umbilical cord blood (UCB). CD34+ cells were obtained via mononuclear cells (MC layer) using a microbeads technology. Shown are representative results to confirm the cells' high purity for CD34 expression and the presence of a significant sub-population of cells lacking CD38 expression. Cells were cultured in 96-well plates containing serum-free medium with various combinations of cytokines for either 2 or 5 days before analysis. (B) Absolute cell counts are shown for cultured cells 5 days after culture initiation. Each well contained medium supplemented with either basal cytokines alone (STF only) or with one of the test cytokines. Cells were counted with Turk staining. Shown are mean values ± SD (n = 3). One-Way-ANOVA test was used for statistical analysis. The value of each group was compared with that for cells given STF only (control group).

A p value < 0.05 was considered statistically significant. Histograms shown in blue reflect values statistically higher than those of the control.

*: P < 0.05, **: P < 0.01, ns: not significant.

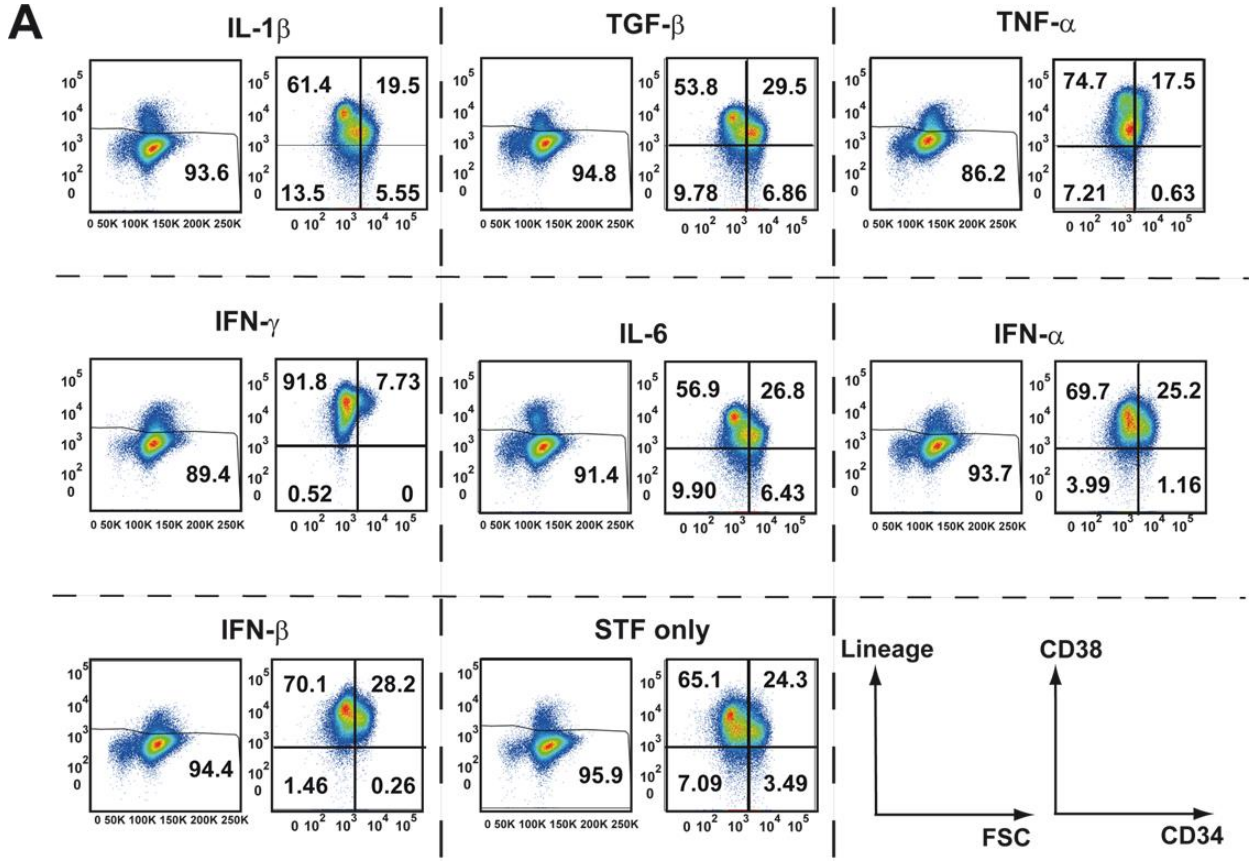
In the culture of HSCs/HSPCs, some cells may self-renew, but others are expected to become committed to differentiation into certain lineages. Cell proliferation should thus be evaluated in combination with differentiation status as well as with markers of a primitive cell phenotype. We therefore examined immunophenotypes of cultured HSCs/HSPCs. To this end, cell surface expression of CD34 and CD38 was investigated together with that of lineage (Lin) markers [12]. When cultured for 5 days under baseline conditions (STF only), most cells (~72.2%) lost CD34 expression. Some cells exhibiting a primitive phenotype persisted (Lin-CD34+CD38-; ~3.5%). Of note is that the effect of each inflammatory cytokine on cell surface marker expression varied highly (Figure 2A). All cytokines decreased the percentage of Lin- cells versus STF only, meaning that all can to some extent promote differentiation (Figure 2B, Lin-). Three cytokines, TNF-α, IFN-γ, and IL-6, enhanced such differentiation particularly strongly (P < 0.0001).

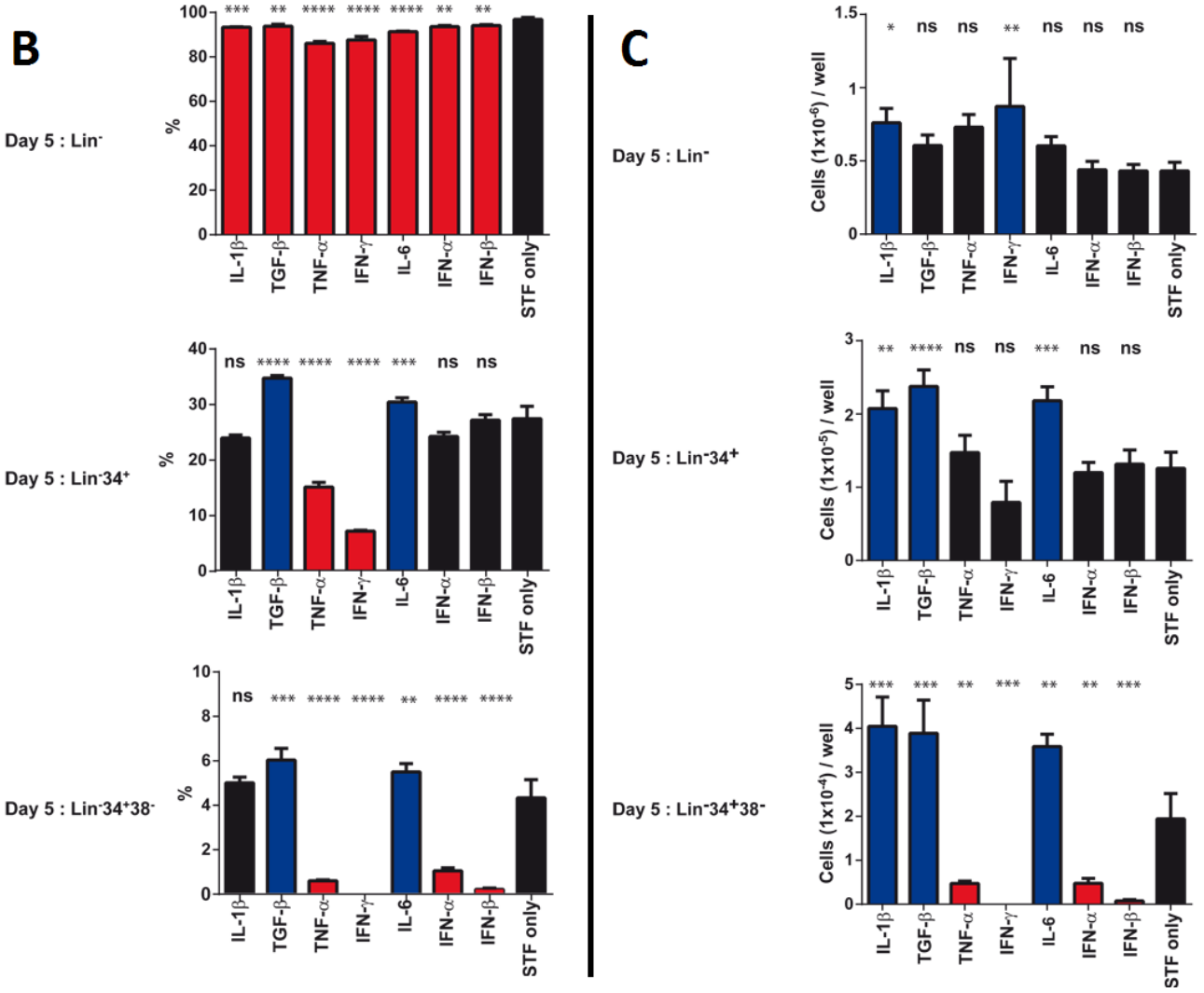
We next focused on how inflammatory cytokines affected preservation of primitive HSC/HSPC populations. As shown in Figures 2B (%) and 2C (absolute numbers), the tested inflammatory cytokines fell into two groups. One enhanced retention of primitive cells (shown in blue when statistical significance was observed) and the other reduced HSC/HSPC preservation (shown in red). Among 3 cytokines in the former group, TGF-β, a key niche molecule capable of in vivo maintenance of HSCs [13], appeared most

potent in supporting primitive cells with minimal loss of Lin- cells, whereas IL-1β and IL-6 expanded primitive cell numbers, but only at the expense of exaggerated differentiation. By contrast, the other 4 cytokines significantly decreased both frequencies (Figure 2B) and numbers (Figure 2C) of Lin-CD34+CD38+ cells. Two type I interferons (IFN-βand IFN-β) exhibited similar effects on differentiation, showing reduction of Lin-CD34+CD38+ cells but no loss of Lin-CD34+ cells (Figures 2B and 2C). In contrast, TNF-α and IFN-γ decreased the frequency of Lin-CD34+ cells (Figure 2B) significantly, indicating that they were more potent in promoting differentiation than were type I interferons. The effect of IFN-γ was striking, with virtually no Lin-CD34+CD38+ cells left in culture, but evoking the highest proliferative response observed in both total (Figure 1B) and Lin- (Figure 2C) cell assessment.

To further illustrate the effect of these cytokines on HSCs/HSPCs, we sought to quantify ROS accumulation in HSCs/HSPCs. ROS are known to be a crucial regulator of HSCs/HSPCs and the level of ROS accumulation likely alters the fate of HSCs/HSPCs. We thus determined ROS levels using HySOx [14] in cells 2 days after culture, to test whether day-2 ROS status predicted end-culture outcomes on day 5. When cultured with IL-1β or TNF-α, the cells' ROS levels increased significantly over those in cells given STF only (Figures 3A and 3B, shown in blue bars). In contrast, IFN-γ culture produced cells with significantly reduced levels of ROS, whereas type I interferons yielded modest decreases in ROS (Figures 3A and 3B). Of note is that despite overall similarity in the effects of TNF-α and IFN-γ (Figures 2B and 2C), their influences on ROS level regulation in HSCs/HSPCs differed sharply (Figure 3B, shown in blue for TNF-α and red for IFN-γ). One must also note that potent stem cell preservers, i.e., TGF-β and IL-6, caused no significant alteration in ROS levels.

**Figure 2:** Inflammatory cytokines modulate differentiation properties of HSCs / HSPCs.






(A) Representative flow cytometry results of cell surface marker assessment on test cells after a 5-day culture. Two images are shown for each culture condition: Lineage markers vs. FSC (forward scatter) plots, left, and CD38 vs. CD34 plots, the right. Figures represent frequency (%) of cells within the corresponding gate. (B-C) Percentages (B) and absolute numbers (C) of indicated cell populations in each culture (day 5) are shown as mean values ± SD (n = 3). Lin-: lineage markers-negative, Lin-CD34+: lineage markers-negative / CD34-positive, Lin-CD34+CD38-: lineage markers-negative / CD34-positive / CD38-negative. One-Way-ANOVA testing was used in statistical analysis. The value of each group was compared with the STF only (control) group using Dunnett's post hoc test. A p value < 0.05 was considered statistically significant. Histograms shown in colors mean statistically higher (blue) or lower (red) values for them than those of control.

**: P < 0.01, ***: P < 0.001, ****: P < 0.0001, ns: not significant.

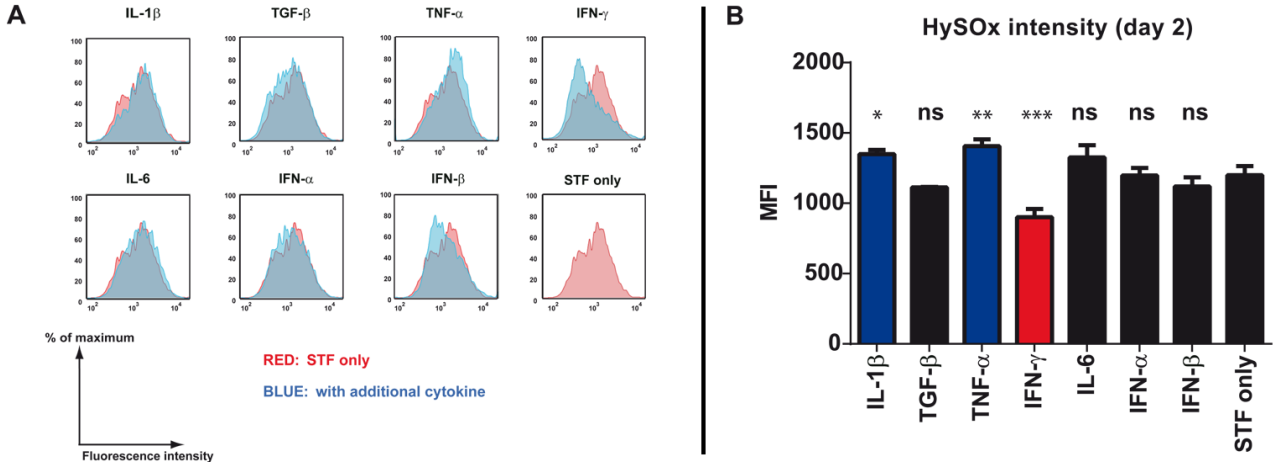
## DISCUSSION

HSCs are sheltered within the BM niche, where they are controlled by diverse signals that maintain their cellular functions. Although the idea that excess of inflammatory cytokines influences the fate of HSCs has been explored, much still remains to be clarified on how inflammation modulates HSCs. Also important is to know how the activity of inflammatory cytokines should be regulated in HSC/HSPC culture to maximize the benefit of ex vivo manipulation of cells for clinical purposes. To this end, we conducted a comprehensive analysis of the effects of 7 key inflammatory cytokines on cultured HSCs/HSPCs, adhering to the essential criterion that different cytokines be compared on the same experimental platform, to exclude any additional variables such as the composition of media, baseline cytokine lot differences, and so on.

When tested in the shared setting of culture with 3 stem cell cytokines (SCF, TPO, and FL), none of the inflammatory cytokines caused massive cell death, thus appearing not to be severely toxic to HSCs/HSPCs. In all culture conditions, including the baseline condition of the 3 test cytokines only,

varying decreases in %Lin- cells were seen, meaning that in all HSC/HSPC differentiation was promoted. Effects on the preservation/expansion of primitive cells (Lin-CD34+CD38-), however, varied significantly among the inflammatory cytokines. Three cytokines, i.e., TGF-β, IL-6, and IL-1β, increased retention of primitive cells, whereas the other 4 cytokines, i.e., two type I IFNs, TNF-α, and IFN-γ, stimulated loss of those cells. A favorable influence of TGF-β on HSC retention is consistent with our previous reports [13, 15]. Positive effects of IL-6 on HSC culture are well known, as exemplified by its use in a cytokine cocktail in clinical trials [16-17] where expansion of human CD34+ cells was the goal. That IL-1β promoted retention of primitive cells is an unexpected observation in light of its reportedly negative effects on HSCs' reconstituting ability [18]. Transplantation experiments will be necessary to prove the utility of IL-1β for HSC expansion. Because technology has recently progressed, with developments such as serum-free culture systems, to re-evaluate these "old" cytokines' effects in HSC culture may be worth while.

**Figure 3:** Inflammatory cytokines variously affect ROS levels 2 days after culture.




(A) Shown are representative results of flow cytometry analysis of ROS levels in 2-day cultured human CD34-positive cells. HySOx was used as an indicator of ROS. (B) Graphic representation of mean fluorescence intensity (MFI) values determined by flow cytometry analysis. Mean values ± SD are shown (n = 3). One-Way-ANOVA testing was used in statistical analysis. The value of each group was compared with those for cells exposed to STF only (control group) using Dunnett's post hoc test. A p value < 0.05 was considered statistically significant. Histograms shown in colors mean statistically higher (blue) or lower (red) values than those in the control group.

*: P < 0.05, **: P < 0.01, ***: P < 0.001, ns: not significant.

Accumulation of ROS is reported to compromise HSC activity [19-21]. On the other hand, recent studies have suggested that ROS play an important role in differentiation of HSCs, especially during the early stages of hematopoietic reconstitution after transplantation [22-23]. These observations suggest that cellular ROS levels should be tightly regulated to maintain in homeostasis the functions of normal HSCs [24]. We accordingly investigated previously unidentified links between inflammatory cytokines and ROS levels in HSCs. In our studies, we used a recently developed ROS indicator, HySOx [14]. We also tested dichlorofluorescein-diacetate (DCF-DA), a widely used probe capable of detecting a wide variety of ROS [25], but discontinued its use because of failure for unknown reasons to obtain consistent results with our specific HSC/HSPC culture system (data not shown). HySOx was originally reported to be suitable for real-time imaging of phagocytes because of its two major properties: 1) Specificity for the single ROS species HOCl, and 2) resistance to autoxidation and to photobleaching. Although interest in relationships between ROS and cellular functions is great, no report has described the cellular roles of individual ROS types, such as superoxide, hydroxyl radical, singlet oxygen, and nitric oxide, as they affect a specific biological phenomenon [11, 26-27]. This is most likely due to a lack of complete set of ROS indicators capable of dissecting each single ROS type from others.

Although the significance of HOCl alone as a phenotypic marker remains uncertain, our results demonstrated that it could predict cellular fates in conjunction with stimulation by inflammatory cytokines. Development of new reagents will help achieve an overview of the impact on cellular functions that each type of ROS has.

In our system, TNF-α and IFN-γ affected HSCs/HSPCs similarly, during 5 days of culture stimulating loss of cells that exhibited primitive cellular immunophenotypes. In contrast, cellular ROS levels 2 days after stimulation differed strikingly, increasing with TNF-α and decreasing with IFN-γ. In fact, previous reports indicate both overlapping and distinct effects on cells for these two cytokines. As reported previously [28-31], an elevated level of TNF-α seems to impair the repopulating capacity of HSCs, possibly by leading them to exhaustion [32]. On the other hand, despite TNF-α being held to disturb homeostasis in HSCs, in vivo approaches using TNF receptor knockout mice led researchers to conclude that the proper level of TNF-α might be necessary for the maintenance of long-time HSC function [33-34]. IFN-γ has been reported to inhibit self-renewal of HSCs while inducing myeloid-biased differentiation [4, 35]. In other reports, chronic stimulation with IFN-γ was shown to trigger BM failure [36], while certain levels of IFN-γ were demonstrated to be required for the proper regulation of HSC activity [37]. In the cases of other cell types, TNF-α, like IL-1 and IL-8, is known to activate dendritic cells, macrophages, and neutrophils, whereas IFN-γ, like IL-2 and type I IFNs, has been shown to activate T cells and NK cells [3].

Close study of immunophenotypes of end-point products found significant differences between cells cultured with TNF-α and cells cultured with IFN-γ (see Figure 2A). While the latter yielded a major population with CD38-bright/CD34-negative phenotype, the former retained a population with CD38-dim/CD34-dim phenotype. This indicates that two cytokines distinctively affected the fates of HSCs/HSPCs for differentiation, despite the stated similarity in their negative effects on preservation of primitive cells. Although further investigation is necessary, we propose that a change in ROS levels early after cultivation may be a determinant of cell fates in differentiation in the context of HSCs/HSPCs and inflammatory cytokines. In contrast, relatively unaltered ROS levels may indicate good culture conditions, such as those with TGF-β, IL-6, and IL-1β, that favor preservation/expansion of primitive cells. As HSCs are predominantly located within BM niches where ROS production is limited at a low level, they thus are protected from ROS stress [22-23]. Our study indicates that inflammatory cytokines affect ROS levels in HSCs/HSPCs, thereby possibly altering cell fates. This notion adds new insights to recent studies that have focused upon a link between inflammation and regulation of HSC functions [3, 38]. More studies should be conducted, and hopefully they will eventually lead to development of strategies to modulate inflammatory cytokines in HSCs or in the surrounding environment as well as to benefit patients suffering from clinical situations where proper regulation of hematopoiesis is required, such as severe infections and some hematological disorders.

## CONCLUSION

Our study demonstrated that inflammatory cytokines altered ROS levels in HSCs/HSPCs in various ways. We also demonstrated the possibility that levels of ROS may indicate cell fate consequences, that is, whether to retain primitive cells or mainly to produce mature cells. Deep comprehension of the relationships between each inflammatory cytokine signal and ROS levels will help in understanding HSCs more precisely. Many kinds of stresses likely alter BM niche dynamics and thereby influence HSCs drastically beyond their compensatory ability. Stress likely also disrupts the normal functions of primitive HSCs. To take these observations into account may assist in better understanding of HSC biology, with amelioration of management for hematopoietic disorders.

## LIST OF ABBREVIATIONS

HSCs – hematopoietic stem cells

HSPCs – hematopoietic stem and progenitor cells

BM – bone marrow

ROS – reactive oxygen species

IL-1β – interleukin-1beta

IL-6 – interleukin-6

TNF-α – tumor necrosis factor-alpha

IFN-α – interferon-alpha

IFN-β – interferon-beta

IFN-γ – interferon-gamma

TGF-β – transforming growth factor-beta

HOCl – hypochlorous acid

MNC – mononuclear cell

SCF – stem cell factor

TPO – thrombopoietin

MFI – mean fluorescence intensity

UCB – umbilical cord blood

Lin – lineage

DCF-DA – dichlorofluorescein-diacetate

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## AUTHORSHIP

T.I., S.Y., and M.O. drafted the manuscript and designed the study. T.I. and S.Y. performed experiments and a statistical analysis.

H.N., M.H., S.Y., and M.O. revised the manuscript and are responsible for the overall design of the study and revision of the manuscript.

## CONFLICTS OF INTEREST

We have nothing to disclose regarding this issue.

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