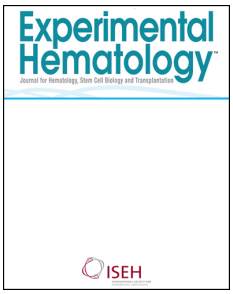

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Proinflammatory signals are insufficient to drive definitive hematopoietic specification of human HSCs in vitro

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Summary

Recent studies in zebrafish and mice demonstrated that proinflammatory signaling is a positive regulator of definitive hematopoietic development. Whether proinflammatory signaling regulates also human hematopoietic specification remains unknown. Here, we explored the impact of the proinflammatory cytokines tumor necrosis factor-α (TNFα), interferon-γ (IFNγ) and interleukin-1β (IL1β) on *in vitro* hematopoietic differentiation using human pluripotent stem cells (hPSCs). Gene expression analysis and ELISA revealed the absence of a proinflammatory signature during hematopoietic development of hPSCs. Functionally, the emergence of hemogenic endothelial progenitors (HEPs; CD31+CD34+CD45- or CD34+CD43-CD73-) and hematopoietic cells (CD43+CD45+) was not affected by treatment with increasing doses of TNFα, IFNγ and IL1β irrespective of the developmental window or the differentiation protocol used (embryoid body- or OP9 coculture-based). Similarly, knock-down of endogenous NF-kB signaling had no impact on hematopoietic differentiation of hPSCs. This study serves as a demonstration that TNFα, IFNγ and IL1β signals do not improve hematopoietic differentiation of hPSCs using current protocols and suggest that proinflammatory signaling is insufficient to drive definitive hematopoietic specification of human HSCs *in vitro*.

Keywords: human PSCs, hematopoietic specification, proinflammatory signals, TNFα, IFNγ, IL1β.

INTRODUCTION

Hematopoiesis is a hierarchical process that is controlled by a rare population of multipotent hematopoietic stem cells (HSCs). *In vivo*, HSCs emerge directly from specialized hemogenic endothelial (HE) cells during development [1-3], a process that is challenging to study in mammals because of the early developmental window in which they arise and the *in utero* location of the embryo [4]. Hematopoietic differentiation of human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) and induced PSCs (hiPSCs), provides a unique opportunity to investigate HSC specification *in vitro [5-7]*; however, *bona fide* HSCs have yet to be efficiently generated from hPSCs, in part due to an incomplete understanding of the multiple signaling pathways that govern HSC fate.

Inflammatory signaling is essential for stress hematopoiesis, either through increasing proliferation or by directing differentiation of HSCs towards myeloid and lymphoid lineages [8, 9]. Early studies suggested that proinflammatory cytokines, such IL1β and IL3, were important regulators of HSC development in the aorta-gonad-mesonephros (AGM) region [10-12]. More recently, a previously unrecognized link was established between inflammatory signaling and definitive hematopoietic emergence, in which tumor necrosis factor-α (TNFα)- and interferon-γ (IFNγ)-mediated proinflammatory signals positively regulated HSC emergence in zebrafish and mouse embryos [13-17]. Although these findings advance our understanding of HSC specification, whether proinflammatory signaling is equally instructive in a human embryonic setting remains unknown [18]. Furthermore, it is unclear whether proinflammatory signals can be used to promote HSC emergence *in vitro* from hPSCs. Here we have explored the impact of TNFα, IFNγ and IL1β cytokines on hemogenic progenitors (HEPs) emergence and their further specification into hematopoietic cells from hPSCs.

EXPERIMENTAL PROCEDURES

Human PSC culture and hematopoietic differentiation

hESC lines H9 (Wicell, Madison, WI) and AND1 (BNLC, Madrid, Spain) and iPSC lines CBI08#4 and CBI08#1 were maintained as described [19]. Approval from the Spanish National Embryo Ethical Committee was obtained to work with hPSCs. For embryoid body (EBs) generation, undifferentiated hPSCs were treated with collagenase IV, scraped off from the Matrigel, transferred to low-attachment plates and incubated overnight in mTESR1 medium (Stem Cell Technologies, Vancouver, Canada) supplemented with bone morphogenetic protein 4 (BMP-4; 50 ng/mL). The following day (day 1), the medium was changed for serum-free defined medium (StemPro-34; Invitrogen) supplemented with monothioglycerol (0.16 µM; Sigma), Holo-transferrin (150 µg/mL), 50 ng/mL BMP-4, and FGFb (10ng/mL). On the third day of differentiation, EBs were changed to differentiation medium (DM) comprising serum-free defined medium (StemPro-34; Invitrogen) supplemented with monothioglycerol, holo-transferrin, 50 ng/mL BMP-4, 300 ng/mL Fms-related tyrosine kinase 3 ligand (Flt-3L), 300 ng/mL stem cell factor (SCF), 10 ng/mL IL-3 and 10 ng/mL IL-6 [20, 21]. TNFα, IFNγ and IL1β were added to cultures at indicated concentrations and refresh during medium changes. To promote definitive hematopoietic program EBs were treated with the GSK3 inhibitor CHIR99021 (3µM) from day 2 to day 3 of culture [22]. All recombinant factors were purchased from R&D Systems. DM was replenished every 3 days.

hPSC lines were also differentiated using OP9 co-culture system as described [23]. hPSC lines were prepared as a suspension of small aggregates using collagenase IV followed by gentle scraping in DM (α-MEM basal medium, 10% non-heat-inactivated FBS, 100 µM monothioglycerol, and 50 mg/mL ascorbic acid) and plated on overgrown

OP9 stroma in 4 mL of DM. On the following day, the medium was replaced with 4 mL of fresh DM to remove unattached cells. From day 3 of co-culture onwards, half-volume media changes were performed every other day. TNFα, INFγ, and IL1β were added to the culture at the indicated doses.

FACS analysis and sorting

Differentiating EBs and hPSC-OP9 co-cultures were dissociated and single cell suspension were stained with anti-CD235a-PE (BD), anti-CD73-BV510 (BD), anti-CD34-PE-Cy7 (BD), anti-CD43-APC (BD), anti-CD34-PE (BD), anti-CD31-APC (Miltenyi), anti-CD45-APC-H7 (BD), 7-AAD, and anti-mouse CD29-FITC (Miltenyi) to exclude OP9 cells from analysis. Live cells identified by 7-AAD exclusion were analyzed using a FACS Canto II cytometer equipped with the FACS Diva analysis software (BD Biosciences). Hemogenic progenitors (HEPs, CD31+CD34+CD45-) hematopoietic progenitor cells (CD34+CD45+) and non-hematopoietic cells (CD31-CD34-CD45-) were purified by FACSAria cell sorter as described [19].

ELISA

Supernatants were collected during EB differentiation and the concentration of TNFα, IFNγ, and IL1β was quantified by ELISA following the manufacturer's instructions (Merck-Millipore).

Western blotting

Differentiating EBs were collected as indicated and treated with 20 ng/mL TNFα for 90 minutes. Western blotting was performed by standard methods. Primary antibodies used were anti-IκBα (Santa Cruz; sc-1643), anti-phospho-IκBα-Ser32/36 (92465) and anti-α-tubulin (both from Sigma).

RNA Purification and Quantitative RT-PCR

Total RNA extraction and quantitative real-time PCR (qPCR) was performed as described [24]. Primer sequences are listed in **Table S1**.

Statistical Analysis

All data are expressed as mean±SD. Statistical comparisons were performed with a paired Student's *t*-test ($P < 0.05$).

RESULTS

hPSC hematopoietic derivatives lack innate proinflammatory phenotype

Proinflammatory signaling positively regulates definitive HSC emergence in vertebrates [13, 15-17], but whether it promotes *in vitro* HSC emergence from hPSCs remains unknown [4]. To address this, we analyzed the impact of TNFα, IFNγ and IL1β on hematopoietic development from two hESCs (H9 and AND1) and two hiPSC lines (CBI08#3 and CBI08#4). Hematopoietic differentiation of hPSCs progresses through two sequential stages: i) specification of hPSCs into hemogenic progenitors (HEPs) and ii) commitment of HEPs into CD45+ hematopoietic cells (**Figure 1A** and **1B,** and **Figure S1A**) [19, 25, 26]. We first analyzed whether hematopoietic cells arising from hPSCs display an innate inflammatory signature. qPCR analysis revealed a very modest upregulation of TNFα receptor 1 (TNFR1) and IFNγ receptor 1 (IFNR1) during EB development, whereas TNFR2 and IL1β receptor (IL1R) were expressed weakly, if at all (**Figure 1C**). To better characterize TNFR1 and IFNR1 expression within developing EBs, their expression was quantified in FACS-sorted HEPs (CD31+CD34+CD45-), CD45+ hematopoietic cells and non-hematopoietic cells (CD31-CD34-CD45-). Both receptors were equally expressed at low levels in the three FACS-purified cell populations (**Figure 1D**). HEPs and CD45+ hematopoietic cells specifically expressed at high levels, the hemogenic marker KDR and the hematopoietic transcription factor PU.1, respectively, confirming the purity/identity of sorted cells. (**Figure S1B**). Moreover, TNFα, IFNγ and IL1β proteins were not present (as detected by ELISA) in EB supernatants at any stage of EB differentiation (**Figure 1E**). Our data indicate that TNFα, IFNγ and IL1β are neither intrinsically produced by hPSC hematopoietic derivatives nor released by neighboring non-hematopoietic cells. Collectively, these results suggest a lack of proinflammatory signature during *in vitro*

hematopoietic development of hPSCs using current protocols and in the absence of infection.

Proinflammatory signaling does not promote hematopoietic emergence during EB differentiation

We next functionally assessed whether exogenous TNFα could promote blood specification from hPSCs. In a first set of experiments, differentiating EBs were cultured with increasing doses of TNFα and the emergence of HEPs (CD31+CD34+CD45-) and further commitment into hematopoietic cells (CD45+) was analyzed by FACS. TNFα treatment failed to promote the emergence of HEPs or hematopoietic cells irrespective of the dose (0, 10, 20 or 40 ng/ml) **(Figure 1F),** hPSC line or the developmental window (EB days 0-7, 7-15 or 0-15) **(Figure S2)**. TNFα-mediated NF-kB activation is tightly regulated in a temporal fashion, with a fast turn-off of its activation occurring after a subtle TNFα pulse [27]. We therefore pulsed differentiating EBs with TNFα (20ng/mL for 24h) at various stages of development and assessed the emergence of HEPs and hematopoietic cells (**Figure 2A**, left panel). Single pulses of TNFα did not impact HEP or hematopoietic cell emergence (**Figure 2A**, right panel). Nevertheless, activation of the NF-kB pathway upon TNFα treatment could be demonstrated during EB development as shown by western blotting of IkBα phosphorylation (**Figure 2B**) [28]. To further determine whether TNFα signaling was relevant for hematopoietic emergence from hPSCs, endogenous TNFα-NF-kB activity was specifically inhibited by BAY65-5811 inhibitor [29]. FACS analysis confirmed that specific inhibition of endogenous NF-kB had no impact on HEPs specification or hematopoietic primitive commitment (**Figure 2C**). IFNγ and ILβ signaling have also been shown to positively regulate blood emergence [10, 17]. We treated developing EBs with increasing concentrations of IFNγ (0, 10, 20 or 40 ng/ml) and ILβ (0, 5, 10

ng/ml) and consistently found no enhancement of hematopoietic emergence (**Figure 2D)**.

Proinflammatory signaling does not promote hematopoietic emergence of hPSCs in OP9 co-culture

We next induced hematopoietic differentiation from hPSCs using an OP9-based co-culture bi-dimensional system **(Figure 3A)** [19, 23]. Exposure of OP9-hPSC co-cultures to increasing concentrations of TNFα, IFNγ or ILβ failed to promote HEPs emergence and CD45+ hematopoietic cells differentiation (**Figure 3B**). Together with the demonstration of NF-kB activation by TNFα during EB development (**Figure 2B**), our data obtained in OP9 co-culture system rule out the possibility that EB impermeability underlies the inability of these ligands to regulate HSC emergence. Collectively, these data demonstrate that TNFα, IFNγ or ILβ ligands do not promote hematopoietic specification of hPSCs irrespective of hPSC type or current differentiation regimen, suggesting that proinflammatory signaling is insufficient to improve HEPs emergence and blood generation from hPSCs.

TNFα and IFNγ do not enhance the emergence of definitive hematopoiesis

In vivo, both TNFα and INFγ have been shown to be dispensable for primitive hematopoiesis but critical for definitive HSC specification [13, 15-17]. To further assess the effect of proinflammatory signaling on the specification of definitive hematopoiesis, developing EBs were treated with the GSK-3 inhibitor CHIR99021 (d2-d3), a well-known Wnt agonist [22, 30-32] coupled with continuous exposure to TNFα (20 ng/mL) or IFNγ (20 ng/mL) and hematopoietic cytokines **(Figure 4A**). We then FACS-analyzed day 9 EBs for several cell surface markers, including CD73 to distinguish hemogenic versus non-hemogenic progenitors [30], CD235a, a marker of primitive hematopoietic progenitors [22], and CD43 known to mark the onset of blood specification [31, 33]. At

this stage of EB development, the majority of the CD31+CD34+ cells were negative for CD43 and CD235a, indicative of definitive hematopoietic program activation (**Figure 4B**) [22]. Consistent with our previous observations, treatment with TNFα and IFNγ failed to enhance HEP (CD34+CD45-CD73- or CD34+CD43-CD235a-) (**Figure 4B**) and definitive hematopoietic cells ($CD34^{dim/+}$CD43+CD45+CD235a-) development (**Figure 4C)**. These findings suggest that proinflammatory signaling is insufficient for driving *in vitro* definitive hematopoietic development from hPSCs.

DISCUSSION

During the last decade, different protocols for hematopoietic differentiation of human (and mouse) PSCs have been developed and have made it possible to routinely produce blood cells [34]. However, the derivation of HSCs with long-term reconstitution potential from hPSCs remains a major goal for regenerative medicine and disease modeling [4, 35]. Proinflammatory signaling has been recently described as a positive regulator of definitive HSC emergence in vertebrates [13, 15-17]. These findings rise the question of whether proinflammatory signaling may positively promote the *in vitro* generation of blood cells from hPSCs. Here we show that exposure to TNFα, IFNγ or IL1β ligands does not potentiate neither primitive nor definitive hematopoiesis from hPSCs, irrespective of the hPSC type, differentiation system or ligand dose/timing treatment. This study shows for the first time that master proinflammatory signals during hPSC hematopoietic differentiation do not enhance blood generation *in vitro* using current protocols.

How to explain this discrepancy in the context of recent *in vivo* data in zebrafish and mice? One possible explanation could be the developmental failure to generate specialized PSC derivatives possessing similar innate immune signature than their *in vivo* counterparts and with the capacity to respond to inflammatory stimuli [13, 15,

16]. This hypothesis is consistent with the fact that we found low or nearly absent expression level of TNFα, INFγ and IL1β receptors during EBs development.

Alternatively, proinflammatory cytokines might need to interact with structural tissue of HSC niche to be effective. This possibility is supported by recent evidence demonstrating that TNFα promotes HSC emergence through activation of Notch pathway within neighboring endothelial cells rather than acting directly on hemogenic progenitors [13, 36]. Although hematopoietic differentiation of hPSCs mimics several aspects of *in vivo* hematopoiesis, the full recapitulation *in vitro* of definitive HSC-supporting microenvironment remains challenging. Mouse fetal liver and AGM-stromal lines have been previously shown to support human hematopoiesis [37-39], and it would be interesting to explore in future studies the effect of proinflammatory signaling on human hematopoietic differentiation from PSCs in co-culture experiments with human fetal liver- and AGM-derived stromal cells. The evolutionary significance of this remains to be determined. It is conceivable that the role of proinflammatory signals in normal HSC development suggests that the hematopoietic system was established, in part, through a response to ancient pathogens [4].

To summarize, in vertebrate embryos proinflammatory signaling promote HSCs emergence. Zebrafish and mouse hematopoiesis share striking similarities to the human blood system, however the full set of required signals for *in vitro* generation of definitive hematopoiesis from hPSCs is still unknown. Together, our data show that blood specification from hPSCs is not enhanced by master proinflammatory signals, suggesting that proinflammatory signaling is insufficient to drive definitive hematopoietic specification of human HSCs *in vitro*, using current protocols.

Conflict-of-interest disclosure: The authors declare no competing financial interests

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FIGURE LEGENDS

Figure 1. Absence of a proinflammatory signature during hematopoietic specification of human PSCs. (A) Schematic diagram of hPSC hematopoietic differentiation during EB formation. *In vitro* hPSC hematopoietic differentiation can be divided into HEPs specification (day 0-10) and hematopoietic commitment (day 10-15). **(B)** Representative flow cytometry analysis identifying HEPs (CD31+CD34+CD45-), total hematopoietic cells (CD45+), and remaining non-hematopoietic (CD31-CD34-CD45-) cell populations at day 15 of EB development. **(C)** Relative expression of TNFR1, TNFR2, IFNR1 and IL1R during hematopoietic differentiation of hPSCs. Peripheral blood mononuclear cells were used as positive control (C+). Data represent the mean±SD of 3 independent experiments (H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). **(D)** Relative expression of TNFR1 and IFNR1 in HEPs, CD45+ hematopoietic and remaining non-hematopoietic FACS-purified cells from day 15 EBs. Data represent the mean±SD of 3 independent experiments (H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). **(E)** Production of TNFα, IFNγ and IL1β cytokines during hPSC hematopoietic differentiation. Supernatants were collected at the indicated time points and analyzed by ELISA. LPS-stimulated PBMNC-conditioned medium was used as a positive control (C+). Data represent the mean±SD of 3 independent experiments (H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). **(F)** hPSC EBs were differentiated into hematopoietic cells in the presence or absence of increasing doses of TNFα (0 to 40 ng/mL), and the percentage of HEPs and total CD45+ blood cells were analyzed by FACS on day 10 (left panel) and day 15 (right panel), respectively. Data represent the mean±SD fold-change relative to the non-treated condition, n=6 independent experiments using H9 hES line, CBI08#3 and CBI08#4 hiPSC lines. Student's t test revealed no statistical significant differences.

Figure 2: Proinflammatory signaling does not promote definitive hematopoiesis from hPSCs during EB differentiation. (A) EBs were pulsed for 24 hours (on days 2, 4, 7 or 10) with TNFα (left panel) and the emergence of HEPs and total CD45+ blood cells was analyzed on day 10 and 15, respectively (right panel). Data represent the mean±SD fold-change relative to the non-treated condition (n=6 independent experiments using H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). Student's t test revealed no statistical significant differences. **(B)** Western blot of phospho- and total IkBα protein in EBs collected at day 2, 7 and 10, and treated with TNFα (20 ng/mL) for 90 minutes. Tubulin was used as a loading control. **(C)** hPSCs were differentiated with or without the specific IKK inhibitor BAY65-1185 at a concentration of 10-20 µM. The percentage of HEPs and total CD45+ blood cells was analyzed by FACS on day 10 and day 15, respectively. Data represent the mean±SD fold-change relative to the non-treated condition (n=6 independent experiments using H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). Student's t test revealed no statistical significant differences. **(D)** hPSC EBs were differentiated into hematopoietic cells in the presence or absence of the indicated doses of IFNγ and IL1β. The percentage of HEPs and total CD45+ blood cells was analyzed by FACS on day 10 and day 15, respectively. Data represent the mean±SD fold-change relative to the non-treated condition (n=6 independent experiments using AND1 hES line, CBI08#3 and CBI08#4 hiPSC lines). Student's t test revealed no statistical significant differences.

Figure 3: TNFα, INFγ and IL1β signaling do not promote definitive hematopoietic differentiation of hPSCs in OP9 co-culture. (A) Schematic representation of hematopoietic differentiation of hPSCs using OP9 co-culture (left panel). Representative plots showing how HEPs and CD45+ cells were quantified within the human CD29- cell population (right panel) [40, 41]. **(B)** hPSCs were differentiated on

OP9 stroma in the presence or absence of the indicated concentrations of TNFα, IFNγ and IL1β. The percentage of HEPs and total CD45+ blood cells was analyzed by FACS on day 8 and day 15, respectively. The data represent mean±SD (n=6 independent experiments using H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). Student's t test revealed no statistical significant differences.

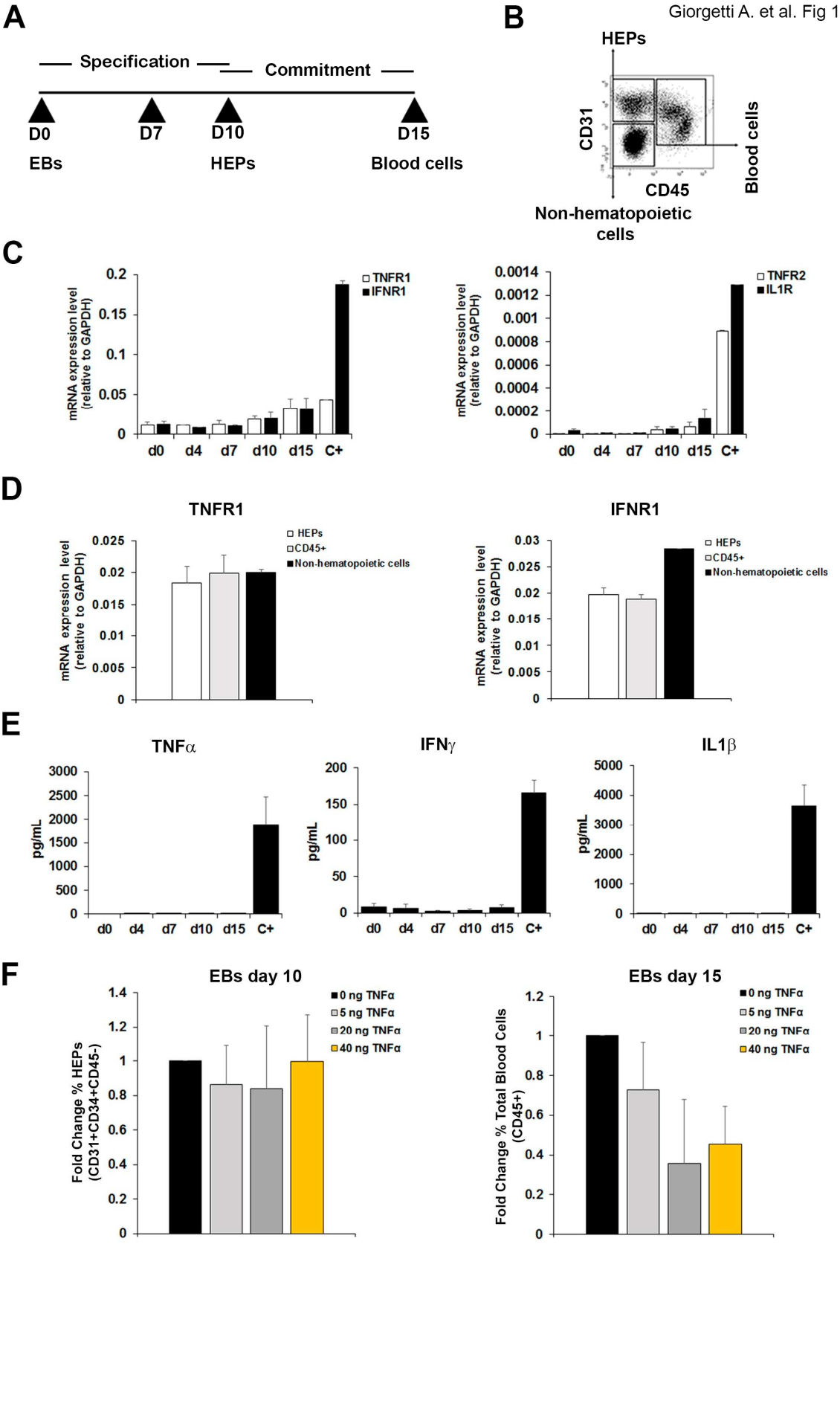
Figure 4: TNFα and INFγ do not promote definitive hematopoietic specification of hPSCs. (A) Scheme showing EB treatment conditions and timeframe used to induce definitive hematopoietic program [22, 30]. EBs were generated and Wnt pathway activation was induced between days 2 and 3 of differentiation by CHIR92011 (3 µM) treatment. CHIR92001 was removed on day 3 and replaced with Flt-3, SCF, BMP4, IL-3 and IL-6 hematopoietic cytokines. **(B)** Flow cytometry analysis of CD34, CD43, CD235a and CD73 expression on day 9 EBs treated with TNFα (20 ng/mL) and (20 ng/mL), n=6 independent experiments using H9 hES line, CBI08#3 and CBI08#4 hiPSC lines. **(C)** Representative flow cytometry analysis for CD34, CD43 and CD45 expression on day 15 EBs treated with 20ng/mL TNFα.

SUPPLEMENTAL FIGURE LEGEND

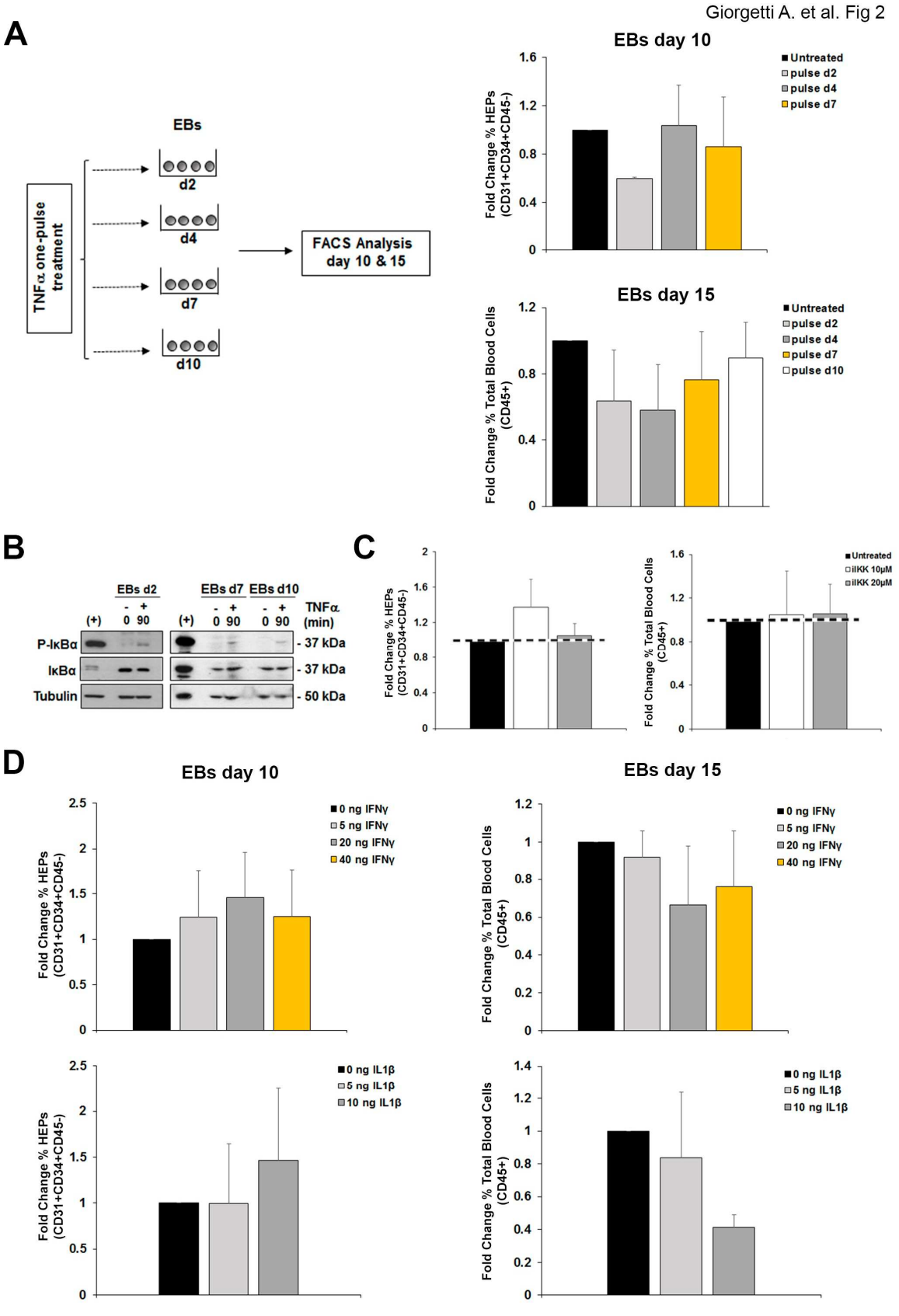
Figure S1: **HEPs and hematopoietic development during EB differentiation. (A)** Kinetics of the appearance of HEPs (CD31+CD34+CD45-) and total blood cells (CD45+) during EB differentiation. Data represent the mean±SD of 3 independent experiments (H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). **(B)** Expression of the HEP marker KDR and the hematopoietic transcription factor PU.1 confirming the identity of the FACS-purified populations. See Figure 1D. Data represent the mean±SD of 3 independent experiments (H9 hES line, CBI08#3 and CBI08#4 hiPSC lines).

Figure S2: The impact of TNFa treatment is not time-dependent. (A) hPSCs were differentiated into hematopoietic cells and treated with TNFα (20 ng/mL) for the indicated developmental windows (days 0-7, days 7-15 and days 0-15). The percentage of HEPs (CD31+CD34+CD45-; left panel) and total blood cells (CD45+; right panel) was analyzed by FACS on day 10 and day 15, respectively. Data represent the mean±SD (n=6 independent experiments using H9 hES line, CBI08#3 and CBI08#4 hiPSC lines). No statistical significant differences were found.

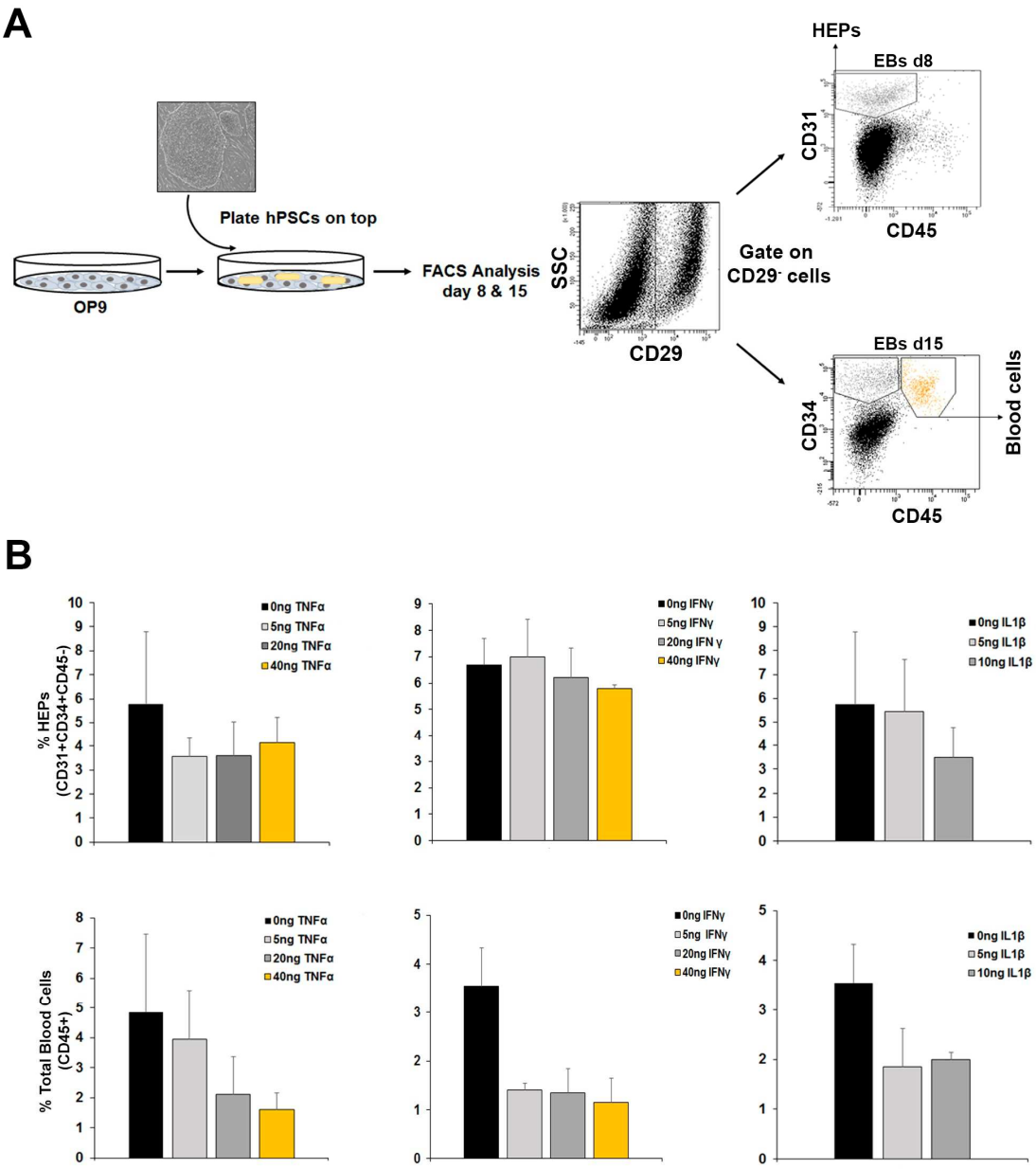
Giorgetti A. et al. Fig 1



Giorgetti A. et al. Fig 2



Giorgetti A. et al. Fig 3



Giorgetti A. et al. Fig 4