Interleukin-1 regulates hematopoietic progenitor and stem cells in the midgestation mouse fetal liver

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ABSTRACT

Background

Hematopoietic progenitors are generated in the yolk sac and aorta-gonad-mesonephros region during early mouse development. At embryonic day 10.5 the first hematopoietic stem cells emerge in the aorta-gonad-mesonephros. Subsequently, hematopoietic stem cells and progenitors are found in the fetal liver. The fetal liver is a potent hematopoietic site, playing an important role in the expansion and differentiation of hematopoietic progenitors and hematopoietic stem cells. However, little is known concerning the regulation of fetal liver hematopoietic stem cells. In particular, the role of cytokines such as interleukin-1 in the regulation of hematopoietic stem cells in the embryo has been largely unexplored. Recently, we observed that the adult pro-inflammatory cytokine interleukin-1 is involved in regulating aorta-gonad-mesonephros hematopoietic progenitor and hematopoietic stem cell activity. Therefore, we set out to investigate whether interleukin-1 also plays a role in regulating fetal liver progenitor cells and hematopoietic stem cells.

Design and Methods

We examined the interleukin-1 ligand and receptor expression pattern in the fetal liver. The effects of interleukin-1 on hematopoietic progenitor cells and hematopoietic stem cells were studied by FACS and transplantation analyses of fetal liver explants, and *in vivo* effects on hematopoietic stem cell and progenitors were studied in $Il1r1^{-/-}$ embryos.

Results

We show that fetal liver hematopoietic progenitor cells express the IL-1RI and that interleukin-1 increases fetal liver hematopoiesis, progenitor cell activity and promotes hematopoietic cell survival. Moreover, we show that in $Il1r1^{-/-}$ embryos, hematopoietic stem cell activity is impaired and myeloid progenitor activity is increased.

Conclusions

The IL-1 ligand and receptor are expressed in the midgestation liver and act in the physiological regulation of fetal liver hematopoietic progenitor cells and hematopoietic stem cells.

Key words: Interleukin-1, hematopoiesis, hematopoietic stem cell, fetal liver, aorta-gonad-mesonephros.

Citation: Orelio C, Peeters M, Haak E, van der Horn K, and Dzierzak E. Interleukin-1 regulates hematopoietic progenitor and stem cells in the midgestation mouse fetal liver. Haematologica 2009; 94:462-469. doi:10.3324/haematol.13728

Acknowledgments: the authors would like to thank all members of the laboratory for helpful discussions and technical assistance, and especially R. van der Linden for expert FACS analysis. We also thank the Erasmus Animal Facility for animal care.

Funding: these studies were supported by NIH R37 DK51077, NWO VICI 916.36.601, Netherlands BSIK Award 03038, KWF Dutch Cancer Society 2001-2442 and HFSP RG0345/1999.

Manuscript received July 29, 2008. Revised version arrived October 27, 2008. Manuscript accepted November 10, 2008.

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The online version of this article contains a supplementary appendix.

Introduction

The adult hematopoietic system consists of at least ten distinct blood cell lineages that are produced through the differentiation of hematopoietic stem cells (HSCs) and many intermediate progenitor cells. In the developing mouse, the first long-term adult repopulating HSCs are found at embryonic day 10.5 (E10.5) in the intra-embryonic aorta-gonad-mesonephros (AGM) region.[1,2] Slightly later, from E11 onwards these HSCs are detected in other hematopoietic tissues, including the fetal liver (FL).[3,4] The FL commences its role as an important embryonic hematopoietic organ at late E9.[5,6] Between E11 and E16 HSC numbers are increased dramatically in the FL and subsequently remain constant until birth,[3,4,7] when the bone marrow takes over as the HSC niche through the adult stages of life. The FL also plays a crucial role in erythropoiesis and hematopoietic progenitor cell expansion.[8-10]

In contrast to the AGM region, which harbors a microenvironment suitable for the generation of HSCs, the FL does not generate HSCs *de novo*, but is thought to be seeded with HSCs from other embryonic sites (i.e. the AGM and/or the yolk sac).[3,5,6,11,12] Subsequent to colonization by hematopoietic cells, the FL provides an excellent *in vivo* environment for HSCs, as demonstrated by the dramatic increase in HSC activity during midgestation.[4,7] Also, several FL stromal cell lines have been shown to maintain and/or expand HSCs *in vitro*, further indicating that the FL contains an HSC supportive microenvironment.[13-15]

Despite the fact that the FL is a pivotal territory for HSCs during development, little is known about the cytokines and growth factors that affect hematopoiesis, and more specifically hematopoietic progenitors and HSCs, within this tissue. Previously, we have shown that the interleukin-1 (IL-1) signaling component TAB2 is expressed in the AGM region at the time of HSC appearance.[16] Additionally, we observed that several IL-1 receptor/signaling components are expressed in the midgestation AGM and that IL-1 increased AGM HSC activity and hematopoiesis.[17] This was a very interesting observation since Il1r$^{-/-}$ adult mice are viable and show no obvious defects in HSC activity or steady-state hematopoiesis.[18,19] Since TAB2, the signaling IL-1 receptor type I and its co-receptor IL-1R associating protein (IL-1RAcP), the decoy IL01R type II and IL-1R signaling components were expressed in the FL, we set out to investigate whether IL-1 may also regulate FL hematopoiesis. It is well-documented that the pro-inflammatory cytokine IL-1 regulates adult hematopoiesis and plays a role in a number of diseases, including autoimmune diseases and leukemia.[20] Besides regulating mature, differentiated hematopoietic cells, functional studies show that IL-1 regulates adult BM HSCs by providing these cells with differentiation and/or proliferation and radio-protective signals.[21-25] Since IL-1 and the IL-1 receptor type I and the co-receptor IL-1RAcP are expressed by BM hematopoietic stem/progenitor cells, it has been suggested that IL-1 acts directly on these immature cells at the base of the hematopoietic hierarchy.[26-28] In this study we examine whether IL-1 plays a role in FL hematopoiesis. We show that the IL-1 receptor type 1 (IL-1RI) is expressed on FL hematopoietic (progenitor) cells and that its ligand IL-1 is also expressed in this tissue. The addition of exogenous IL-1 to FL explants increases hematopoietic progenitor activity and the overall number of hematopoietic cells but does not alter HSC activity. However, when analyzing Il1r1$^{-/-}$ mice, we observed that FL HSC activity was severely impaired, while myeloid progenitor activity was increased. Hence, IL-1 appears to be a complex regulator of hematopoiesis in the FL.

Design and Methods

Embryo generation and cell culture and progenitor assay

Animals were housed according to institutional guidelines, with free access to water and food. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals. Matings for embryo generation were between (CBAxC57BL/10) F1 females and Ln72 human β-globin[29] males, C57BL/6 females and Act-GFP males,[30] C57BL/6 males and females, and Il1r1$^{-/-}$ (Il1r$^{tm1Imx/\,tm1Imx}$)[18] males and females. The day of the vaginal plug was counted as day 0. Pregnant mice were sacrificed, embryos isolated, and livers were dissected[31] and resuspended in PBS/10% FCS/1% Pen/Strep as single cells.

Cells from 3-4 pooled fetal livers were seeded at 0.5×10^4 to 7.5×10^4 cells per plate in methylcellulose medium (Methocult GF M3434; Stem Cell Technologies Inc.) containing SCF, IL-3, IL-6 and Epo and incubated at 37°C, 5% CO_2. Colony-forming unit-granulocyte, macrophage, granulocyte macrophage and granulocyte erythroid megakaryocyte macrophage (CFU-G, -M, -GM and –GEMM respectively) and burst-forming unit-erythroid (BFU-E) were scored with an inverted microscope at day 7 of culture.

RNA isolation, cDNA synthesis and RT-PCR analysis

Total RNA was isolated with TRIZOL, cDNA generated with Superscript II reverse transcriptase (Invitrogen/ Life Technologies), and PCR reactions performed with Amplitaq (PerkinElmer) as previously described.[17] PCR primer sequences are listed in the *Online Supplementary Table S1*.

Immunohistochemistry

Embryos were snap-frozen in TissueTek (Sakura). 7-10 μM cryosections were fixed in 2% paraformaldehyde/PBS and stained with IL-1α antibody (clone 12A6; BD Biosciences) as previously described.[17] Pictures were taken with an Olympus BX40 microscope (Olympus Nederland B.V., Zoetewoude, NL) using an Olympus lens at 20x/0.40 PH and 40x. Images were acquired and processed with Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA, USA).

Organ cultures and in vivo *transplantation assays for hematopoietic stem cell activity*

E11 liver tissues (marked with GFP, human β-globin) were dissected and 2-3 day organ cultures were performed in the presence of 0, 1 or 10 ng/mL IL-1β (TebuBio). To inhibit IL-1 signaling, 50 ng/mL IL-1Ra (R&D systems) or 100 ng/mL IL-1a blocking antibody (R&D systems) was added to the cultures. After culture, single cell suspensions were obtained and different cell dilutions (measured as embryo equivalents) were injected intravenously together with non-marked $2x10^5$ spleen cells into 9.5 Gray irradiated (CBAxC57BL/10)F1 or (129SvxC57BL/6)F1 recipient mice. E11 liver tissues were isolated from *Il1r1*$^{+/+}$ and *Il1r1*$^{-/-}$ E11 male embryos and injected intravenously together with $2x10^5$ normal female spleen cells into 9.5 Gray irradiated C57BL/6 female recipient mice. Repopulation was assayed at one and four months post-transplantation by donor specific PCR (human β-globin, GFP, Y chromosome gene) on peripheral blood DNA as previously described.[31,32] Only mice with >10% engraftment were considered repopulated. For multi-lineage repopulation analysis, DNA was isolated from spleen, thymus, bone marrow and peripheral blood or from FACS-sorted cells from these tissues and assayed for donor contribution by PCR.

FACS analysis

Single cell suspensions were stained with IL-1RI-PE antibody (clone 35F5, Becton Dickinson) on ice for 30 minutes. Cells were co-stained with FITC labeled antibodies for c-kit (CD117), Mac-1 (CD11b), CD45 or CD31 (Pharmingen). Dead cells were excluded by 7AAD (Molecular Probes) and FACS analysis was performed on FACScan (Becton Dickinson). Other FACS analyses were performed with FITC-anti-Mac1 (CD11b), PE-anti-CD34 (RAM34), APC-anti-c-kit (CD117) (clone 2B8) or PerCPCy5.5-anti-CD45 (BD) antibodies. Dead cells were excluded by Hoechst 33258 (1 mg/mL, Molecular Probes). Analysis was performed on FACS Aria (Becton Dickinson) and with Cell Quest software.

Statistical analysis

Data are expressed as mean ± SEM. Differences were considered to be significant at $p<0.05$ as analyzed with the Student's *t* test.

Results

Fetal liver hematopoietic cells express IL-1RI

Previously we observed IL-1 ligand and receptor RNA expression in E11-E12 FL tissues.[17] Moreover, IL-1-induced gene expression and signaling in E11-E12 FL cells indicated that the IL-1 pathway was functional. To examine which cell types could be affected by IL-1, flow cytometric analysis for IL-1RI expression was performed. On average, 5.1% of E11.5 and 3.5% of E12.5 FL cells (Table 1) express the IL-1RI. As the total number of FL cells increases 7.6 fold between E11.5 and E12.5 ($4.9x10^5±1.0x10^5$ and $37x10^5±11x10^5$ respectively), there is a 5.2 fold increase in the absolute number of FL IL-1R+ cells, from $0.24x10^5$ to $1.26x10^5$. Multi-parameter flow cytometric analysis with the pan-hematopoietic marker CD45 revealed that E11.5 and E12.5 FL contain on average 1.1% and 0.3% IL-1RI$^+$CD45$^+$ cells respectively (Figure 1A and Table 1), indicating that 22% and 9% of the IL-1RI$^+$ population is hematopoietic in E11.5 and E12.5 FL cells respectively. Moreover, 1.3% and 0.7% of E11.5 and E12.5 FL cells respectively are IL-1RI$^+$c-kit$^+$, suggesting that 22% to 27% of the IL-1RI$^+$ are hematopoietic progenitor/stem cells. This is supported by flow cytometric analysis demonstrating that some FL IL-1RI$^+$ cells express CD31 and Mac1 (*data not shown*), markers of FL HSCs and myeloid cells.[4,33]

In addition to the expression of the IL-1RI, the ligand IL-1β is expressed in discrete small patches of cells as shown by immunostaining of E12.5 FL (Figure 1B). Flow cytometric analysis revealed that 0.04-0.12% of FL cells express the IL-1α ligand (*data not shown*). Thus, both IL-1 expressing cells and putative IL-1 responsive cells are present in the fetal liver.

Table 1. Percentages of IL-1RI$^+$ hematopoietic cells in E11 and E12 fetal liver.

	E11-11.5 FL		E12-12.5 FL	
	Mean %	(range)	Mean %	(range)
IL-1RI$^+$CD45$^-$	4.0	3.5-4.5	3.2	1.8-4.2
IL-1RI$^+$CD45$^+$	1.1	0.7-1.5	0.3	0.1-0.6
IL-1RI$^-$CD45$^+$	11.6	10.1-15.3	6.6	5.6-7.0
IL-1RI$^+$c-kit$^-$	3.5	3.0-4.3	2.5	1.7-2.8
IL-1RI$^+$c-kit$^+$	1.3	1.1-1.7	0.7	0.2-1.1
IL-1RI$^-$c-kit$^+$	22.9	15.3-39.4	12.5	9.9-14.4

Combined IL-1RI flow cytometric data from several independent experiments in which E11-E11.5 FL (n=5) and E12-E12.5 FL (n=4) from age-matched littermates were pooled for analysis.

Interleukin-1 increases the number of fetal liver hematopoietic cells

To determine whether IL-1 affects hematopoietic cells, FL explants cultured in the presence or absence of IL-1 were examined by flow cytometric analysis. As shown in Figure 2A, IL-1 increases the percentage of FL CD45$^+$, c-kit$^+$ and Mac1$^+$ cells in a dose-dependent manner. Addition of 1 ng/mL IL-1 increased percentages 1.4 to 1.6 fold, and 10 ng/mL IL-1 significantly increased these populations 1.7 to 2.3 fold as compared to control cultures. Since IL-1 did not affect total cell numbers in cultured liver explants (*Online Supplementary Figure S1*), the absolute numbers of CD45$^+$, c-kit$^+$ and Mac-1$^+$ cells were similarly increased.

Blocking experiments with a natural IL-1 receptor antagonist IL-1Ra (which binds to the IL-1RI but does not evoke receptor signaling) or an IL-1α specific blocking antibody were performed to test whether hematopoietic cell increases were specific to IL-1 signaling. IL-1Ra significantly decreased the percentage of CD45$^+$ and c-kit$^+$ cells in the cultures by an average of 0.8 and 0.7 fold respectively (Figure 2B), as did the IL-1α blocking antibody *(data not shown)*. Thus, endogeneous IL-1 allows specific expansion of FL hematopoietic cells in *ex vivo* tissue cultures.

Interleukin-1 affects on fetal liver gene expression and apoptosis

The consequences of IL-1 mediated signaling in the FL (gene expression changes) were monitored by RT-PCR for components of the IL-1 signaling pathway, other cytokine genes and cell survival-related genes. The gene expression levels of IL-1 signaling components and cytokine genes, *Csf1* (M-CSF), *Csf3* (G-CSF) and *Kitl* (SCF) did not change in the FL explants cultured in the presence of IL-1 (*Online Supplementary Figure S2*). However, as shown in Figure 3A, while the expression of anti-apoptotic genes, *Bcl2* and *Slugh* in the FL were unaffected by addition of 1 and 10 ng/mL of IL-1 as compared to uncultured FL or FL explants cultured without IL-1, *Bcl2l1* (*Bcl-x*) gene expression was up-regulated in an IL-1 dose dependent manner. The pro-apoptotic *Bax* and *Bim* genes were unaffected by IL-1 addition. Thus, IL-1 could be influencing the viability of FL hematopoietic cells through the modulation of apoptotic pathways.

To test this, flow cytometric analysis for the pre-apoptotic marker, AnnexinV, in combination with CD45 and c-kit was performed on FL explants. After 2 days of culture, the percentage of AnnexinV$^+$ cells in the CD45$^+$ FL cell population was significantly decreased in the presence of IL-1 (Figure 3B). The percentage of apoptotic cells in the c-kit$^+$ FL cell population was similarly decreased in the presence of IL-1 (*data not shown*). Thus, IL-1 affects FL hematopoietic cells by promoting cell survival.

Interleukin-1 increases fetal liver hematopoietic progenitor activity

The effects of exogenously added IL-1 on FL HSCs was tested by *in vivo* transplantation experiments. Cells from E11.5 liver explants cultured in the absence or presence of 1 or 10 ng/mL IL-1β were injected into irradiated adult mice and examined one and four months post-transplantation. As shown in Figure 4A, the low dose of IL-1β (1 ng/mL) but not the high dose (10 ng/mL) increased the percentage of repopulated mice at one month post-transplantation, indicating that IL-1β increases short-term repopulating hematopoietic pro-

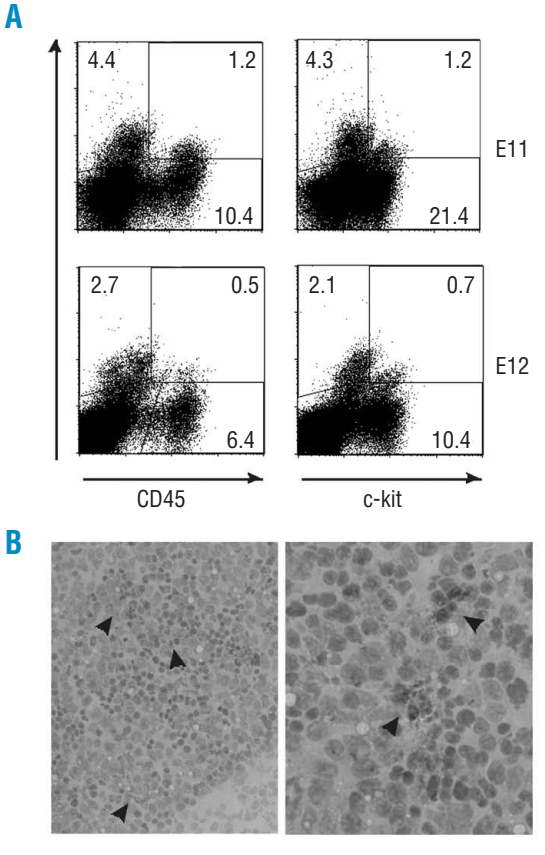

Figure 1. IL-1 Receptor and IL-1 are expressed in the FL. (A) Multiparameter flow cytometric plots of E11 and E12 fetal liver cells showing IL-1RI, CD45 (hematopoietic cell marker) and c-kit (hematopoietic stem/progenitor marker) expression. Percentages of single positive and double positive cells are indicated in the quadrants. Approximately 80,000 events are shown. (B) Immunohistochemistry for IL-1β protein expression on transversal E12 embryo trunk sections. IL-1β expression can be detected in discrete cell clusters indicated by the arrowheads. Cells indicated by the arrowheads in the right panel appear to be stained in the cytoplasm of large flattened cells. Final magnification, 200x (left) and 400x (right).

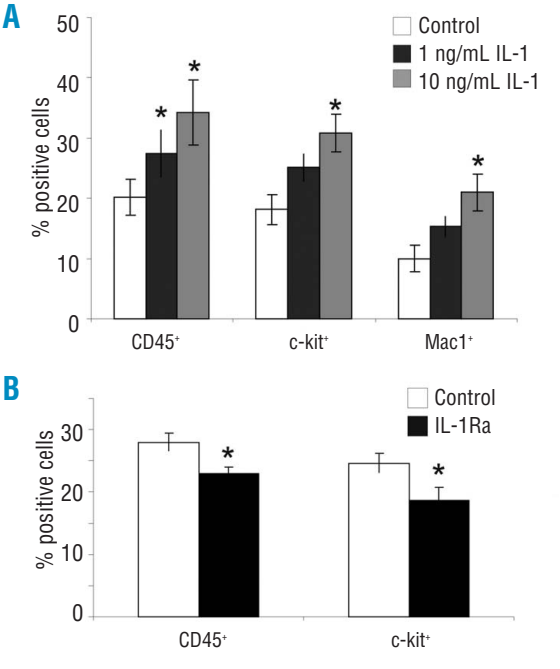

Figure 2. IL-1β increases hematopoiesis in the E11 FL explants. E11 FL tissues were cultured for three days as explants in the absence or presence (1 or 10 ng/mL) of IL-1β or IL-1R antagonist (IL-1Ra). After this culture period, single cell suspensions were generated, cell counts were performed (*Online Supplementary Figure S1*) and the percentage of hematopoietic (CD45$^+$), or hematopoietic progenitor/stem cells (c-kit$^+$ or Mac1$^+$) was determined by flow cytometric analysis. (A) Percentages of CD45$^+$, c-kit$^+$ and Mac1$^+$ cells after FL explant culture in increasing doses of IL-1β. The bars indicate the average percentage of marker positive cells from 5 independent experiments and the error bar indicates the SEM. Asterisks (*) indicate statistical significance. (B) IL-1R antagonist (IL-1Ra) addition decreases the percentage of CD45$^+$ and c-kit$^+$ cells in FL explant cultures. The bars indicate the average percentage of marker positive cells from 3 independent experiments and the error bars indicate the 2xSEM. Asterisks (*) indicate statistical significance.

genitor activity in the FL. In contrast, at four months post-transplantation, neither dose of IL-1 affected long-term repopulating HSC activity in the FL. The same percentages of engrafted recipients were found as with control FL cultured in the absence of IL-1. Chimerism levels were high and engraftment was multilineage (Figure 4B). Thus, exogenously added IL-1 increases short-term FL hematopoietic progenitor activity in FL explants, and does not affect long-term FL HSC activity.

Interleukin-1 receptor deficiency affects fetal liver hematopoietic progenitors and stem cells

To determine if IL-1 plays a role in FL hematopoiesis in a more physiological setting, we analyzed FLs from E11 *Il1r1*$^{-/-}$ embryos for hematopoietic progenitor and HSC activity. The absolute number of *Il1r1*$^{-/-}$ FL cells ($6.14\times10^5\pm7.0\times10^4$ cells) is significantly increased (1.3 fold) as compared to wild type FL cells ($3.7\times10^5\pm2.5\times10^4$ cells; n=6-9). Flow cytometric analyses showed that although the percentage of FL CD45$^+$ cells in *Il1r1*$^{-/-}$ embryos (8.3%) was slightly lower as compared to wild type embryos (11.4%), the absolute number of CD45$^+$ FL cells was similar ($4.0\text{-}4.2\times10^4$). Also, no differences were found in the absolute number of cells expressing Mac1 ($1.8\times10^4\pm9.2\times10^3$ in *Il1r1*$^{+/+}$ FL; $1.8\times10^4\pm2.0\times10^3$ in *Il1r1*$^{-/-}$ FL) or c-kit ($2.5\times10^5\pm2.1\times10^4$ in *Il1r1*$^{+/+}$ FL; $2.8\times10^4\pm1.7\times10^4$ in *Il1r1*$^{-/-}$ FL). Interestingly, the absolute number of CD34$^+$Mac1$^+$c-kit$^+$ cells (phenotypic HSCs) was 1.3-fold decreased and the absolute number of Gr1+ myeloid (progenitor) cells was 2.1-fold increased in the *Il1r1*$^{-/-}$ FL as compared to wild type FL (Figure 5A).

In clonogenic progenitor assays (Figure 5B), BFU-E numbers were decreased by 1.9-fold in the *Il1r1*$^{-/-}$ FL. CFU-M and CFU-G were 1.9-fold and 1.3-fold increased in *Il1r1*$^{-/-}$ FL. CFU-GM, the common progenitor for CFU-M and CFU-G, showed no significant (1.2-fold) increase in the *Il1r1*$^{-/-}$ FL, but a striking 2.5-fold decrease was found for the CFU-GEMM, the most immature multipotent progenitor. Together with the phenotypical analyses, these progenitor activity data suggest that IL-1 signalling *in vivo* differentially affects mature myeloid progenitors and immature multi-potent hematopoietic progenitors.

The effect of *in vivo* FL IL-1 signaling on HSC activity was examined by long-term transplantation studies with

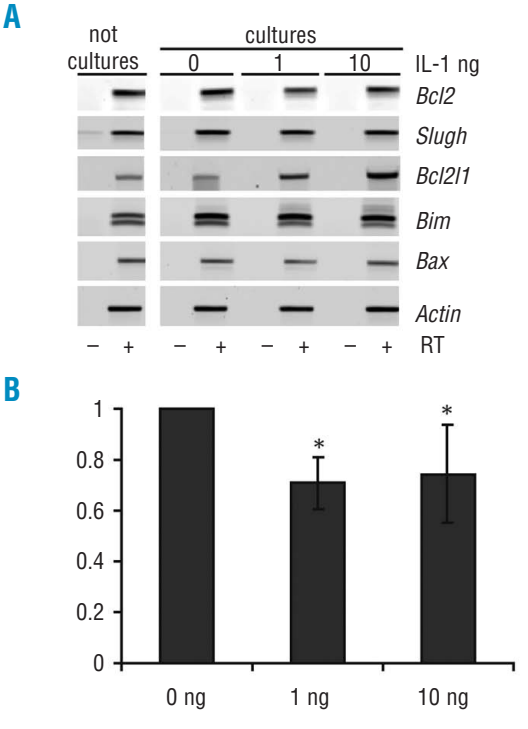

Figure 3. IL-1 affects apoptosis-related gene expression levels and hematopoietic apoptosis in FL explants. E11.5 FL tissues were directly analyzed or analyzed after culture for three days in the presence or absence of 1 or 10 ng/mL IL-1β. (A) RT-PCR gene expression analysis for the anti-apoptotic genes Bcl-2 (*Bcl2*), *Slugh* and Bcl-x (Bcl2l1) and the pro-apoptotic *Bax* and *Bim* genes. Expression was normalized against the housekeeping gene actin. (B) FACS analysis of E11.5 FL explants for apoptotic marker AnnexinV. Bar graph indicates the fold induction of the average percentage of AnnexinV$^+$ (apoptotic) cells within the hematopoietic (CD45$^+$) FL cell population after two days of explant culture in the presence or absence of IL-1. Error bars indicate the SEM (n=3) and asterisks (*) indicate statistical significance. RT=reverse transcriptase.

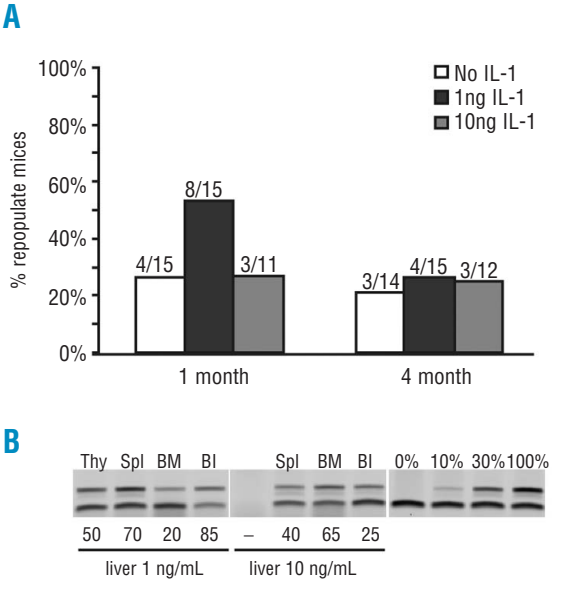

Figure 4. IL-1β increases short-term, but not long-term HSC activity in FL tissues. E11.5 FL explants were cultured in the absence or presence of 1 or 10 ng/mL IL-1β. Single cell suspensions were obtained and injected into lethally irradiated recipients. At one and four months post-transplantation the peripheral blood was tested for donor contribution to the hematopoietic system by semi-quantitative PCR. (A) Combined results from 7 independent experiments in which one embryo equivalent (ee) of cultured FL cells were injected per recipient. Bars indicate the percentage of repopulated mice per condition after one and four months post-transplantation. The numbers above the bars indicate the number of repopulated recipients out of the total number of injected recipients. SE ranged from 10-13% and the one month result with 1 ng IL-1 shows a statistically significant difference from the other repopulation results. Only mice with >10% donor chimerism were considered repopulated and multi-lineage repopulation was confirmed in several recipients. (B) Representative multi-lineage analysis for 2 recipients that were injected with FL cells cultured in the presence of 1 or 10 ng/mL of IL-1. All hematopoietic tissues analyzed by semi-quantitative PCR show high levels of donor contribution, confirming that IL-1 does not compromise the ability of the FL HSCs to allow multi-lineage reconstitution of the recipient.

freshly isolated and three day cultured *Il1r1*$^{-/-}$ and Il1r1$^{+/+}$ FLs. As shown in Figure 5C, direct transplantation of *Il1r1*$^{-/-}$ FL cells resulted in fewer repopulated mice (13%) as compared to the number repopulated with wild type cells (71%). After three days of culture, HSC activity in *Il1r1*$^{+/+}$ FL explants was reduced (20%) compared to HSC activity in directly isolated FL demonstrating the suboptimal conditions of FL explant cultures for supporting HSCs.

Despite the suboptimal conditions, the *Il1r1*$^{-/-}$ FL explants did not experience further significant losses in HSC activity. Thus, the lack of *in vivo* IL-1R1 signaling in the FL leads to decreased immature hematopoietic progenitor (CFU-GEMM) and HSC numbers, and a concomitant increase in CFU-M.

Discussion

We have shown here that the well-known adult pro-inflammatory cytokine IL-1 plays a role in the regulation of FL hematopoietic cells, progenitors and HSCs during midgestation development. Both the IL-1 ligand and the signal transducing IL-1R type I and IL-1RAcP, as well as the IL-1R type II decoy receptor are expressed by E11-E12 FL hematopoietic cells and progenitors. In *ex vivo* FL explant cultures exogenously added IL-1 induces an increase in hematopoietic cell numbers and these increases are IL-1 dose dependent. Thus, FL hematopoietic cells are sensitive to IL-1. IL-1 also increases short-term repopulating hematopoietic progenitor numbers in *ex vivo* FL explant cultures. However, increases were found at 1 ng/mL and not 10 ng/mL of IL-1, indicating that subsets of hematopoietic cells are differentially sensitive to IL-1 dose.

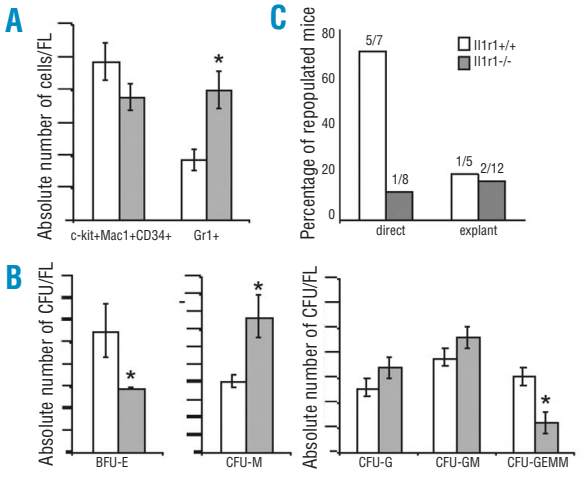

Figure 5. IL-1R deficiency affects FL hematopoietic progenitors and HSCs *in vivo*. E11 FLs were dissected from wild type (*Il1r1*$^{+/+}$; white bars) or *Il1r1*$^{-/-}$ (gray bars) embryos and assayed. (A) The absolute number of c-kit$^+$Mac1$^+$CD34$^+$ cells and Gr-1$^+$ cells in *Il1r1*$^{+/+}$ and *Il1r1*$^{-/-}$ FL as determined by flow cytometric analysis. Percentage marker positive cells and total viable FL cells were used to calculate absolute cell numbers per FL. Asterisk (*) indicates statistical significance. Results represent the average ± SEM of at least 5 experiments. (B) Progenitor numbers per *Il1r1*$^{+/+}$ and *Il1r1*$^{-/-}$ FL as determined by methylcellulose clonogenic assay. Total numbers of BFU-E, CFU-M, CFU-G, CFU-GM and CFU-GEMM per FL are shown. Colony numbers are derived from triplicate cultures scored after seven days of culture (n=3, cells from 14 *Il1r1*$^{+/+}$ and 15 *Il1r1*$^{-/-}$ FLs). Asterisks (*) indicate statistical significance. (C) Percentage of adult recipient mice repopulated with HSCs from *Il1r1*$^{+/+}$ and *Il1r1*$^{-/-}$ FLs either directly transplanted (direct) or transplanted after three days of explant culture (explant). Numbers of mice repopulated/number of mice transplanted are indicated. Only mice with more than 10% donor chimerism were considered repopulated. Statistically significant differences are found in the repopulation results between *Il1r1*$^{+/+}$ direct and *Il1r1*$^{+/+}$ explant, and between *Il1r1*$^{+/+}$ direct and *Il1r1*$^{-/-}$ explants.

However, neither FL explants cultured with exogenously added IL-1 nor IL-1R deficient FL explants were changed in HSC activity. In fact, HSC activity in wild type FL explants is vastly decreased as compared to freshly isolated FL tissue,[3,5,6,11,12] indicating that FL explants (in contrast to AGM explants) do not have the appropriate microenvironment for autonomous *ex vivo* HSC maintenance or expansion. Hence, on its own, IL-1 cannot mediate FL HSC expansion *ex vivo*.

Developmental similarities in IL-1 mediated hematopoietic cell regulation

Recently, we showed that IL-1 plays a role in limiting the progenitor differentiation in the midgestation AGM and that IL-1R deficiency results in decreased AGM HSC activity.[17] Compared to the AGM region, the levels

To examine more carefully the affects of IL-1 signaling on hematopoietic cells, we analyzed IL-1RI deficient embryos for FL hematopoietic defects. Directly analyzed E11 *Il1r1*$^{-/-}$ FL revealed decreases in the absolute number of c-kit$^+$Mac1$^+$CD34$^+$ phenotypic HSCs (1.3-fold) and CFU-GEMM immature hematopoietic progenitors (2.5-fold). We found that HSC activity is severely (3.3-fold) decreased. Taken together, IL-1 signaling *in vivo* appears to contribute to the expansion and/or maintenance of most FL HSCs. As expected, based on the absence of a steady state hematopoietic phenotype in *Il1r1*$^{-/-}$ adult mice,[18] the *Il1r1*$^{-/-}$ FL was not completely deficient in HSC activity, suggesting that IL-1 is not absolutely required. Further studies should provide insight into the IL-1 independent HSC subset that apparently provides for the relatively normal hematopoiesis found in adult *Il1r1*$^{-/-}$ mice. A more careful analysis of HSC activity, number and self-renewal capacity of *Il1r1*$^{-/-}$ adult mice could in future studies provide a better insight into HSC regulation by inflammatory cytokines.

In contrast to the HSCs and immature progenitors (CFU-GEMM), the number of CFU-M and Gr-1$^+$ cells was increased in the *Il1r1*$^{-/-}$ FL. These data suggest an additional role for IL-1 signaling *in vivo*, perhaps acting to limit myeloid cell expansion. FACS analysis of *Il1r1*$^{-/-}$ FL cells for CMP, GMP, MEP and CLP should provide insight into which specific cell in the lineage hierarchy is affected. Our data do not allow discrimination as to whether IL-1 would act directly on IL-1RI expressing hematopoietic progenitors or indirectly on the cells of the FL microenvironment (4.4% of FL cells are CD45-IL-1RI$^+$; Figure 1A) that in turn affect hematopoietic progenitors. Alternatively, IL-1 signaling may limit the differentiation of HSC to myeloid progenitors *in vivo*. Initially we used the *ex vivo* explant culture system to examine these possibilities.

of IL-1RI expression, as well as the percentages of IL-1RI$^+$ cells are higher in the E11-E12 FL. Despite the fact that the AGM and the FL are rather different tissues, both in tissue architecture and cellular composition, there are striking similarities between IL-1 mediated hematopoietic (stem and progenitor) cell regulation. Most interestingly, both HSC and progenitor activity are decreased in *Il1r1*$^{-/-}$ AGM and FL, while the myeloid progenitors are increased, suggesting a similar role for IL-1 signaling in these two embryonic hematopoietic microenvironments.

Also like in the AGM region, IL-1RI is not exclusively expressed on CD45$^+$ FL hematopoietic cells. In the adult BM, we found that more than 99% of the IL-RI$^+$ cells are CD45$^+$ (*Orelio et al., unpublished data, 2008*). Approximately 60-90% of FL IL-1RI$^+$ cells do not express the CD45 or c-kit hematopoietic markers. Interestingly, we observed IL-1RI and IL-1RAcP expression (by RT-PCR and FACS) in several AGM and FL derived stromal cells.[17] Immunostainings of AGM tissues localize IL-1RI expression to both hematopoietic and mesenchymal cell regions. Despite the almost exclusive expression of IL-1RI on adult BM hematopoietic cells, the adult BM stromal cell line FBMD-1 also expresses IL-1RI and IL-1RacP.[17] Thus, the expression of IL-1RI on both hematopoietic and non-hematopoietic cells suggests that IL-1 plays a role in regulating hematopoiesis in both a direct and an indirect manner in both AGM and FL. While IL-1 may act, in a limited manner, on the adult BM microenvironment (less than 1% of the IL-R1$^+$ cells are CD45$^-$), the high percentages of IL-1RI$^+$ non-hematopoietic cells in FL and AGM strongly suggest that these embryonic microenvironments are responsive to IL-1 and consequently affect hematopoiesis.

A role for IL-1 in regulating apoptosis of fetal liver hematopoietic cells

The IL-1 mediated increase in hematopoietic (progenitor) cells in FL explant cultures suggests that IL-1 may influence cell survival. Previously we and others have shown that apoptosis plays a role in regulating HSCs in the AGM, FL and BM.[34-36] RT-PCR analysis revealed that the anti-apoptotic genes *Bcl2* and *Bcl2l1* (*Bcl-x*) were down-regulated in AGM tissues cultured in the presence of IL-1, but flow cytometric studies with Annexin V staining showed no consistent effect of IL-1 on the viability of c-kit$^+$ cells (*Orelio et al., unpublished data, 2008*). In contrast, IL-1 did increase the expression level of the anti-apoptotic *Bcl2l1* gene in FL explants and decreased apoptosis in the FL hematopoietic (progenitor) population. Other studies have shown that mice deficient in IL-1R signaling components, such as TAB2 and NFκB pathway components (i.e. p65 NFκB and IKK) are embryonic lethal, due to severe FL degeneration caused by apoptosis.[37-40] Also, the IL-1R signaling component TAK1 is required for BM hematopoietic cell survival.[41] Taken together, these results suggest that IL-1 receptor signaling contributes to maintaining the balance between the life and death of FL and BM hematopoietic cells. This is further supported by many previous studies of *in vivo* IL-1 administration in which IL-1 has been shown to provide radioprotection to BM hematopoietic progenitors and stem cells.[24,25,42]

In conclusion, we have shown here that the pro-inflammatory cytokine IL-1 and its receptor are expressed in the midgestation FL and that IL-1 can act as a regulator of HSCs and of myeloid differentiation in this tissue. One of the possible mechanisms by which IL-1 may regulate these hematopoietic cells is via cell survival. Also, enhanced differentiation of HSCs and progenitors (resulting in decreased HSC and progenitor numbers), could contribute to increased myeloid progenitor numbers. Thus, this study reveals an exciting role for an adult cytokine in regulating the immature hematopoietic cells during FL development.

Authorship and Disclosures

CO: contributed to conception and design, analysis and interpretation of data; to drafting the article and revising it critically for important intellectual content; and approval of this version; MP contributed to conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content; and approval of this version; EH: contributed to conception and design, analysis and interpretation of data; to drafting the article and revising it critically for important intellectual content; and approval of this version: KvdH: contributed to analysis and interpretation of data; to drafting the article ; and approval of this version; ED: contributed to conception and design, analysis and interpretation of data; to drafting the article and revising it critically for important intellectual content; and approval of this version.

The authors reported no potential conflicts of interest.

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