Deficiency in interferon type 1 receptor improves definitive erythropoiesis in Klf1 null mice

Maria Francesca Manchinu1 · Carla Brancia2 · Cristian Antonio Caria1 · Ester Musu1 · Susanna Porcu1 · Michela Simbula1 · Isadora Asunis1 · Lucia Perseu1 · Maria Serena Ristaldi1

Received: 21 April 2017 / Revised: 21 September 2017 / Accepted: 29 September 2017 / Published online: 11 December 2017
© The Author(s) 2017. This article is published with open access

Abstract
A key regulatory gene in definitive erythropoiesis is the transcription factor Krüppel-like factor 1 (Klf1). Klf1 null mice die in utero by day 15.5 (E15.5) due to impaired definitive erythropoiesis and severe anemia. Definitive erythropoiesis takes place in erythroblastic islands in mammals. Erythroblastic islands are formed by a central macrophage (Central Macrophage of Erythroblastic Island, CMEI) surrounded by maturating erythroblasts. Interferon-β (IFN-β) is activated in the fetal liver’s CMEI of Klf1 null mice. The inhibitory effect of IFN-β on erythropoiesis is known and, therefore, we speculated that IFN-β could have contributed to the impairment of definitive erythropoiesis in Klf1 knockout (KO) mice fetal liver. To validate this hypothesis, in this work we determined whether the inactivation of type I interferon receptor (Ifnar1) would ameliorate the phenotype of Klf1 KO mice by improving the lethal anemia. Our results show a prolonged survival of Klf1/Ifnar1 double KO embryos, with an improvement of the definitive erythropoiesis and erythroblast enucleation, together with a longer lifespan of CMEI in the fetal liver and also a restoration of the apoptotic program. Our data indicate that the cytotoxic effect of IFN-β activation in CMEI contribute to the impairment of definitive erythropoiesis associated with Klf1 deprivation.

Introduction
Klf1 is essential for definitive erythropoiesis in mice [1, 2] and man [3] and is a key regulator of many erythroid genes [4, 5]. Klf1 KO mice are strongly anemic and die by E15.5 [1, 2]. Early reports indicated that progenitor cells fail to undergo terminal erythroid differentiation in Klf1−/−embryos and latter stage erythropoiesis is compromised [6, 7]. We previously described a non-erythroid specific target of Klf1, the endonuclease enzyme DNaseII-alpha of CMEI [8]. Definitive erythropoiesis takes place in erythroblastic islands in mammals. Erythroblastic islands are highly specialized hematopoietic tissue sub-compartments that play a critical role in regulating erythropoiesis. Erythroblastic islands contain a CMEI surrounded by erythroid cells at different stage of maturation [9–12]. The central macrophage plays several roles in erythroid maturation including the engulfment of extruded nuclei from erythroblasts and degradation of the nuclear DNA by DNase II alpha. Dnase2a KO mice have a peculiar erythroid phenotype. The mice die around E17.5 of lethal anemia, which is caused by IFN-β production by CMEIs, which contain a large amount of undigested DNA [13]. IFN-β produced in the fetal liver inhibits the erythropoiesis that occurs in association with macrophages at the erythroblastic island. Undigested DNA directly stimulates CMEIs to express IFN-β and, consequently, interferon-responsive genes, which inhibit erythropoiesis by apoptosis and kill the embryos [13–16].

Similarly, we have previously shown that IFN-β is activated in the fetal liver of Klf1 KO embryos [8]. This observation suggested to us that IFN-β could have contributed to the Klf1 KO phenotype, exacerbating the defect in definitive erythropoiesis.

Edited by R De Maria

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41418-017-0003-5) contains supplementary material, which is available to authorized users.
We determined whether the inactivation of Ifnar1 would ameliorate the phenotype of Klf1 KO mice by improving the lethal anemia. Here, we show that Klf1/Ifnar1 double KO embryos survive longer in utero. The longer lifespan is accompanied by an improvement of definitive erythropoiesis. Erythrocytes undergo terminal erythroid differentiation and enucleate more efficiently, although with a reduced efficiency compared to WT mice. The amelioration of definitive erythropoiesis in double KO mice compared to the single Klf1 null littermates is accompanied by an increased number of functional CMEIs in the fetal liver, which indicates a prolonged survival of the erythroblastic islands. The apoptotic program, perturbed in the fetal liver of Klf1 null embryos, is more similar to the WT control. This improvement in definitive erythropoiesis, however, is not sufficient for the complete rescue of Klf1 null embryos that are nevertheless strongly anemic and die by E16.5.

These data may contribute to better understand the role of Klf1 in definitive erythropoiesis. Our results could also have implications on strategies for β-hemoglobinopathy therapies that target transcription factors involved in γ-globin repression [17, 18] such as Klf1 [19]. In addition, the knowledge of the precise pathophysiology of Klf1 shortage may improve the counseling and therapy of the pathologies caused by Klf1 mutations, including fetal hydrops [20].

### Results

#### Partial rescue of Klf1 KO mice

In this study, we aimed to determine if the type I interferon response contributes to the lethal anemia of the Klf1 null phenotype by inactivating IFN-β in CMEIs. To this end we crossed Klf1+/- mice with mice deficient in Ifnar1, which...
IFNAR1 deficiency improves Klf1 null mice erythropoiesis

Definitive erythropoiesis and enucleation are improved in Klf1−/− Ifnar1−/− mice fetal liver

To investigate whether the ameliorated phenotype of Klf1−/− Ifnar1−/− compared to Klf1−/−Ifnar1+/+ embryos was accompanied by an improvement in the fetal liver erythropoiesis, we analyzed, by flow cytometry, E14.5 fetal liver cells. Ifnar1 expression is not affected by Klf1 deprivation in the E14.5 fetal liver (see Supplementary Information).

Klf1−/−Ifnar1++ and Klf1−/−Ifnar1−/− mice lack Ter119 expression (see Supplementary Information) [6, 22]. To date, CD44 transcription has not been reported to be influenced by Klf1 deprivation in mouse fetal liver cells [22]. However, CD44 is reported to be affected by Klf1 in human erythrocytes [23–25]. To determine if the expression of CD44 gene is affected by the absence of Klf1 in mouse, using flow cytometry, we compared, CD44 expression level in WT and Klf1−/−Ifnar1++ fetal liver cells from E14.5 samples (Fig. 2a). Klf1 KO fetal liver cells showed a clearly detectable expression of CD44. Therefore, CD45 and PI negative fetal liver cells were analyzed with CD44 and cell size as markers [23, 24] (Fig. 2b). Proerythroblasts (CD44+Ter119low) were excluded from the analysis since the lack of Ter119 hampers their unambiguous identification [26, 27].

We considered three different populations: Population II (PII), represented by basophil erythroblasts (big cell size, CD44++), Population III (PIII), represented by polychromatic erythroblasts (medium cell size, CD44+++) and Population IV (PIV), orthochromatic erythroblasts (small cell size, CD44++) (Fig. 2b) [26, 27]. The same genotypes were analyzed by hematoxylin/eosin staining on E14.5 fetal liver slides (Fig. 2c).

First, we compared the pattern of differentiation among WT and Klf1−/−Ifnar1−/− cells. Flow cytometry highlights an identical expression profile (Fig. 2b) and no morphological differences in the histologic examination of hematoxylin/eosin fetal liver (Fig. 2c), demonstrating that no alteration had taken place due to the lack of Ifnar1 response during erythropoiesis.

Analysis of Klf1−/−Ifnar1++ mice fetal liver cells by flow cytometry showed higher heterogeneity compared to the WT in terms of cell size and CD44 level of expression (Fig. 2b). Hematoxylin/eosin staining showed a general impairment of the tissue (Fig. 2c) with the increase of interstitial space between cells (arrows) compared to other genotypes and likely alteration of the extracellular matrix. A predominance of hematopoietic cells can be seen in the liver from WT mice. The density of these cells is similar in the Klf1−/−Ifnar1+/− liver. In the Klf1−/− Ifnar1++ liver, cell density is reduced and several cells showed degenerative changes like condensed nuclei.

IFNAR1 deficiency improved Klf1 null mice erythropoiesis

Since Ifnar1−/− mice develop normally [21], we generated and intercrossed Klf1+/−Ifnar1−/− mice. As a control, we intercrossed Klf1−/−Ifnar1−/− mice.

Genotype analysis of 130 newborn mice from Klf1+/−Ifnar1−/− intercrosses indicated that Ifnar1 deficiency does not rescue the in utero lethality caused by Klf1 absence.

To verify the possibility of a partial rescue with a prolonged in utero survival of the embryos we evaluated the embryos from E14.5 onwards (Table 1). At E14.5 the homozgyous Klf1−/−Ifnar1+/+ is severely anemic (Fig. 1a). Compared to the WT littermates, the embryo was smaller (75.42 ± 8.2% SD 8.2%, p ≤ 0.001), extremely pale and no blood vessels are visible (Fig. 1a, b) [1, 2]. Otherwise, embryos with a double deficiency for Klf1 and Ifnar1 were equal in size to the Klf1+/−Ifnar1−/− littermates (p = 0.64), and although paler, some blood vessels were visible (Fig. 1a, b). Klf1−/−Ifnar1+/+ were also significantly smaller than Klf1−/−Ifnar1−/− and Klf1+/−Ifnar1−/− embryos (p ≤ 0.001) (Fig. 1b). On the other hand no statistically significant difference in size was detected among Klf1+/−Ifnar1−/−, Klf1−/−Ifnar1−/− and Klf1++/Ifnar1−/− embryos (Fig. 1b).

Klf1−/−Ifnar1−/− fetal livers appear bigger and less pale compared to Klf1−/−Ifnar1+/+ fetal livers (Fig. 1a and data not shown).

To assess the potential improvement in fetal liver cellularity we normalized the number of E14.5 fetal liver cells of each genotype to that of Klf1+/+/Ifnar1+/+ embryos (Fig. 1c). The cellularity of Klf1−/−Ifnar1−/− was more than 35% (0.57 ± 1.0 – 0.47 ± 1.0 SD 0.001) higher than that of Klf1−/−Ifnar1−/− (0.23 ± 0.10 – 0.23 ± 0.10 SD 0.012), however it was significantly lower than Klf1+/−Ifnar1−/− (0.90 ± 0.30 – 0.90 ± 0.30 SD 0.056) embryos (Fig. 1c; p ≤ 0.001). No statistically significant difference was detected between Klf1+/−Ifnar1−/− and Klf1+/−Ifnar1+/+ embryos (Fig. 1c).

This result indicates an improvement of the fetal liver development in Klf1−/−Ifnar1−/− compared to Klf1−/−Ifnar1+/+ embryos.

At E15.5 there was an improvement in the survival of Klf1−/−Ifnar1−/− embryos with respect to Klf1−/−Ifnar1+/+. Klf1−/−Ifnar1−/− embryos were paler than Klf1+/−Ifnar1−/− littermates but otherwise developmentally normal and alive. At this stage, the Klf1+/−Ifnar1−/− were all dead (no heartbeat) [1]. By day 16.5 Klf1−/−Ifnar1−/− embryos were dead (no heartbeat) (Table 1).

These data demonstrate a prolonged in utero survival of the Klf1−/−Ifnar1−/− embryos compared to the Klf1−/−Ifnar1+/+ embryos, accompanied by an improvement of fetal liver development.

encoded the common receptor for IFN-α and IFN-β. IFN-α is not activated in Klf1−/− mice fetal liver [8].

Since Ifnar1−/− mice develop normally [21], we generated and intercrossed Klf1+/−Ifnar1−/− mice. As a control, we intercrossed Klf1−/−Ifnar1−/− mice.

Genotype analysis of 130 newborn mice from Klf1+/−Ifnar1−/− intercrosses indicated that Ifnar1 deficiency does not rescue the in utero lethality caused by Klf1 absence.

To verify the possibility of a partial rescue with a prolonged in utero survival of the embryos we evaluated the embryos from E14.5 onwards (Table 1). At E14.5 the homozgyous Klf1−/− Ifnar1+/+ is severely anemic (Fig. 1a). Compared to the WT littermates, the embryo was smaller (75.42 ± 8.2% SD 8.2%, p ≤ 0.001), extremely pale and no blood vessels are visible (Fig. 1a, b) [1, 2]. Otherwise, embryos with a double deficiency for Klf1 and Ifnar1 were equal in size to the Klf1+/−Ifnar1−/− littermates (p = 0.64), and although paler, some blood vessels were visible (Fig. 1a, b). Klf1−/− Ifnar1+/+ were also significantly smaller than Klf1−/−Ifnar1−/− and Klf1+/−Ifnar1−/− embryos (p ≤ 0.001) (Fig. 1b). On the other hand no statistically significant difference in size was detected among Klf1+/−Ifnar1−/−, Klf1−/−Ifnar1−/−, and Klf1++/Ifnar1−/− embryos (Fig. 1b). Klf1−/−Ifnar1−/− fetal livers appear bigger and less pale compared to Klf1−/−Ifnar1+/+ fetal livers (Fig. 1a and data not shown).

To assess the potential improvement in fetal liver cellularity we normalized the number of E14.5 fetal liver cells of each genotype to that of Klf1+/+/Ifnar1+/+ embryos (Fig. 1c). The cellularity of Klf1−/−Ifnar1−/− was more than 35% (0.37 ± 1.0 – 0.47 ± 1.0 SD 0.001) higher than that of Klf1−/−Ifnar1−/− (0.23 ± 0.10 – 0.23 ± 0.10 SD 0.012), however it was significantly lower than Klf1+/−Ifnar1−/− (0.90 ± 0.30 – 0.90 ± 0.30 SD 0.056) embryos (Fig. 1c; p ≤ 0.001). No statistically significant difference was detected between Klf1+/−Ifnar1−/− and Klf1+/−Ifnar1+/+ embryos (Fig. 1c).

This result indicates an improvement of the fetal liver development in Klf1−/−Ifnar1−/− compared to Klf1−/−Ifnar1+/+ embryos.
In Klf1−/-Ifnar1−/− KO samples lacking Ifnar1 expression, flow cytometry analysis highlighted less cellular heterogeneity and had a plot more similar to the WT (Fig. 2b). Hematoxylin/eosin staining demonstrated a substantial improvement of the fetal liver architecture. Klf1−/-Ifnar1−/− liver histology resembled that of WT mice with restoration of extracellular matrix. The density of the hematopoietic cells was similar to that of Klf1+/+Ifnar1−/− and WT liver (Fig. 2c).

Quantitation of the three erythroblast populations among WT and Klf1+/+Ifnar1−/− showed no differences in the pattern of differentiation (Fig. 2d). Quantitative comparison between Klf1−/-Ifnar1−/−, Klf1−/-Ifnar1+/−, and WT fetal liver cell populations showed a significant difference in the three erythroblast populations and highlighted a partial recovery of the normal kinetic of the erythropoiesis in the Klf1−/-Ifnar1−/− fetal liver compared to Klf1−/-Ifnar1+/− mice (Fig. 2d). Populations II, III and IV remained virtually flat in Klf1−/-Ifnar1+/− fetal liver (PII: 30.3% +/- SD 1.4; PII: 37.9% +/- SD 1.3 and PIV: 32.2% +/- SD 2.8) while in Klf1−/-Ifnar1−/− fetal liver they showed a progressive increase (PII: 19.7% +/- SD 0.57; PII: 30.6% +/- SD 4.4 and PIV: 53.4% +/- SD 7.5). These percentages are significantly different from the Klf1 null mice as well as from the WT control (PII: 6.1% +/- SD 0.48; PII: 25.3% +/- SD 1.2 and PIV: 68.6% +/- SD 1.4) (Fig. 2d) but showed a partial restoration of the normal erythropoiesis where erythroblast differentiation is coupled to proliferation, which is missing in the Klf1−/-Ifnar1+/− fetal liver.

Cytospin analysis of Klf1−/-Ifnar1−/− fetal liver cells revealed the presence of the entire repertoire of erythroblasts population (Fig. 2e) including late stage enucleating orthochromatric erythroblasts (Fig. 2e, black head arrow) and enucleated reticulocytes (Fig. 2e, black arrow). Cytospin of Klf1−/-Ifnar1−/− fetal liver cells evidenced the presence of the three erythroblastic populations but no reticulocytes were detected (Fig. 2e), confirming recent reports [7]. We quantified the proportion of enucleating orthochromatric erythroblasts and reticulocytes from cytospin in fetal liver of the different genotypes. We identified as “enucleating orthochromatric erythroblasts” cells with an elongated shape and with an asymmetric or protruding nucleus. As shown in Fig. 2f, the percentage of enucleating orthochromatric in Klf1−/-Ifnar1−/− fetal liver was significantly decreased compared to WT (4.8% +/- SD 2.4 vs. 9.3% +/- SD 1.7; p = 0.026). On the other hand the proportion of enucleating orthochromatric was re-established in Klf1−/-Ifnar1−/− fetal liver (11.1% +/- SD 1.4; p = 0.14) and was significantly different from the Klf1−/-Ifnar1−/−+ fetal liver (p = 0.023). Furthermore, reticulocytes could also be detected in the fetal liver (Fig. 2e), although at a reduced number compared to the WT (1.8% +/- SD 0.2 vs. 11.4% +/- SD 1.6; p = 0.0023) (Fig. 2g), while they are undetectable in Klf1−/-Ifnar1−/−+ fetal liver (Fig. 2g).

Cytospin of peripheral blood confirmed the presence of enucleated cells in Klf1−/-Ifnar1−/− mice (Fig. 2h), which are absent in Klf1−/-Ifnar1−/−+ peripheral blood (Fig. 2h).

A number of erythroid genes targeted by Klf1 were also analyzed by RT-qPCR (see Supplementary Information). None of the analyzed genes was increased to a level that may have contributed to the observed improvement in definitive erythropoiesis.

In summary, flow cytometry, hematoxylin/eosin staining, and cytospin analysis demonstrated an improvement of Klf1 KO erythropoiesis, with partial restoration of enucleation in mice lacking Ifnar1 expression.

Improvement in fetal liver CMEI

In Klf1 null mice, CMEI are strongly compromised in number and shape and IFN-β is activated [8]. We wished to evaluate if the observed improvement of definitive erythropoiesis in Klf1−/-Ifnar1−/− could be associated with an improvement of CMEI. Immunofluorescence analysis using F4/80 showed the distribution and morphological features of macrophage cells in mouse fetal liver (Fig. 3a). Interestingly, when we quantified the distribution of macrophage populations, we observed a significant increase of the average number per field of F4/80 positive macrophages in Klf1−/-Ifnar1−/− compared to Klf1−/-Ifnar1−/+, (18.2 +/- SD 2.21 vs. 13.6 +/- SD 0.28, respectively; p = 0.022) (Fig. 3b). Quantitative assessment of F4/80 positive cells by

Fig. 2 Improvement of fetal liver definitive erythropoiesis. a Histograms representing flow cytometry of CD44 labeled freshly isolated fetal liver cells from E14.5 mice showing the comparison between Klf1+/+Ifnar1+/+ (upper histogram) and Klf1−/-Ifnar1−/− (lower histogram) level of expression of CD44 marker. b FACS analysis of normoblastic population of E14.5 fetal liver cells on Klf1+/+Ifnar1+/+, Klf1+/+Ifnar1−/−, Klf1−/-Ifnar1+/+, and Klf1−/-Ifnar1−/− genotypes. Populations II, III, and IV are labeled as PII, PIII, and PIV and are evidenced by blue, orange, and purple color respectively. c Hematoxylin/eosin staining of E14.5 fetal liver slides from same genotypes as in b. Arrows indicate intestinal space. Magnification: 20X. d Percentages of P II, III, IV, of same genotypes as in b from CD44 Vs FSC flow cytometry analysis (p-value: * ≤ 0.05; ** ≤ 0.01). e Cytospin images of fetal liver cell suspensions from same genotypes as in b. Black head arrow indicating an enucleating orthochromatric normoblastic, black arrow indicating a reticulocyte. Magnification: 60X. f Bar chart representing percentage of fetal liver enucleating orthochromatric erythroblast from cytospin of Klf1+/+Ifnar1+/+ (22 fields, a total of 637 cells) Klf1−/-Ifnar1−/− (51 fields, a total of 1385 cells), and Klf1−/-Ifnar1−/− (41 fields a total of 1080 cells), genotypes (p-value: * ≤ 0.05). g Quantification of percentage of reticulocytes from cytospin of Klf1+/+Ifnar1+/+ (39 fields, a total of 807 cells), Klf1−/-Ifnar1−/− (44 fields, a total of 929 cells), and Klf1−/-Ifnar1−/− (62 fields, a total of 1310 cells) fetal liver cells (p-value: ** ≤ 0.01; *** ≤ 0.001). h Cytospin of peripheral blood from same genotypes as in b (black arrow indicate enucleated cells). Magnification: 40X.
FACS confirmed the increased number of macrophages in Klf1-/-Ifnar1-/- compared to Klf1-/-Ifnar1+/+ (see Supplementary Information).

In our previous study [8], we observed two morphologically different types of fetal liver macrophages. In mice with the Klf1+/+ genotype, we identified cells showing typical morphology, with extensive cytoplasmic projections (stellate macrophages) [28, 29] while smaller and rounder macrophages without cytoplasmic protrusions (non stellate macrophages), were mainly observed in Klf1-/- fetal livers.

In this work, we identified an increased proportion of macrophages showing normal morphology, with extensive cytoplasmic projection, in Klf1-/-Ifnar1-/- compared to Klf1-/-Ifnar1+/+ (Fig. 3a). The ratio between macrophages with a classical stellate morphology in comparison to non-stellate morphology was almost double in Klf1-/-Ifnar1-/- compared to Klf1-/-Ifnar1+/+ E14.5 fetal liver (0.50 +/- SD 0.06 vs. 0.26 +/- SD 0.04, respectively, p = 4.6 x 10^-3), (Fig. 3c).

These results suggest that the improved definitive erythropoiesis in Klf1-/-Ifnar1-/- compared to Klf1-/-Ifnar1+/+ embryos is associated with prolonged survival of the CMEI and of the associated erythroblastic islands.

**Improvement of the apoptotic program**

Apoptotic mechanisms play a relevant role in the control of erythropoiesis [30, 31]. Recent evidence has shown that the apoptotic gene expression pattern is perturbed in the absence of Klf1 [32]. It has been suggested that the distorted apoptotic genetic program may contribute to the failure of terminal erythroid differentiation in Klf1 null mice [32]. In particular, Caspase-3 has been shown to be activated in Klf1 null erythroid cells [32]. We have carried out western blotting analysis of whole-cell E14.5 fetal liver protein extract of WT, Klf1+/+, Klf1-/-Ifnar1-/-, and Klf1-/-Ifnar1-/- embryos using a specific antibody for activated murine Caspase-3 and Actin as loading control. As shown in Fig. 4a, only in Klf1-/-Ifnar1+/+ fetal liver cells is there evidence of the 17 and 19 KD forms of activated Caspase-3, which are absent in the other genotypes, including Klf1-/-Ifnar1-/-.

We looked for additional hallmarks of improvement of the apoptotic program. We carried out a TUNEL assay on E14.5 CD71 + fetal liver erythroid population. As shown in Fig. 4b and c, a significant increase of the percentage of apoptotic cells (6.3% +/- SD 0.55) is observed in Klf1-/-Ifnar1+/+ embryos compared to the other genotypes (p < 0.001). It is notable that the percentage of TUNEL positive
cells is decreased in Klf1-/-Ifnar1-/- embryos (1.96 ± 0.13) to a level not significantly different from WT and Klf1+/+Ifnar1-/- control embryos (1.88% ± 0.14 and 2.06% ± 0.21; respectively).

Moreover, we have analyzed by RT-qPCR, the expression level of the anti-apoptotic Bcl2l1 gene, suggested to be a target of Klf1 [33] and downregulated in Klf1 null fetal liver embryos [32], and of the pro-apoptotic Bcl2l11 gene, a critical determinant of erythroid cell survival that is upregulated in Klf1 null fetal liver embryos [32]. As shown in Fig. 4d, e, a significant down regulation of Bcl2l11 (0.55 ± 0.11) and up regulation of Bcl2l11 (1.79 ± 0.016) in Klf1-/-Ifnar1-1/ embryos compared to the WT (p ≤ 0.001) are observed, in agreement with previous results [32]. On the other hand no significant difference in the expression level of these two genes was been detected among Klf1-/-Ifnar1-1/-, Klf1+/+Ifnar1-1/- and WT embryos (Fig. 4d, e).

These results indicate a significant re-establishment of the normal apoptotic program in Klf1 null mice lacking the indicated. Cells were co-stained for TUNEL and CD71. c Bar chart representing the percentage of cells staining TUNEL positive in the four genotypes as indicated. d Expression level of the anti-apoptotic Bcl2l1 gene; genotypes are indicated in the histogram. e Expression level of the pro-apoptotic Bcl2l11 gene; genotypes are indicated in the histogram.

Interferon type I receptor. These data support the notion that Interferon-β response, through Ifnar1, contributes to the perturbation of the apoptotic program seen in Klf1 null mice.

Discussion

The most obvious effect of Klf1 deprivation in mice is lethal β-thalassemia [1, 2]. However, rescue of globin imbalance does not rescue the lethal phenotype, suggesting a major role of Klf1 in definitive erythropoiesis [34].

In our previous work, we have shown that Dnase2 is strongly downregulated in the fetal liver of Klf1 KO mice and that IFN-β is activated in CMEI [8]. The inhibitory effect of IFN-β on erythropoiesis has been known for decades [35]. Here, we reasoned that the absence of the Ifnar1 gene, by hampering the activation of Interferon-β response in the fetal liver, could lead to an amelioration of the Klf1 null phenotype in mice. To verify our hypothesis we
established with a significant kinetic of fetal liver erythropoiesis is partially re-established with a significant progressive increase of the populations is expected. In Klf1 null mice. Terminal erythroid differentiation is a process through which proliferation is linked to differentiation. For every mitosis, distinct populations of erythroblasts are produced. Proerythroblasts undergo three mitoses to sequentially generate basophilic, polychromatophilic, and orthochromatophilic erythroblasts. In normal murine terminal erythroid differentiation, a kinetic of a progressive increase of the populations is expected. In Klf1 null mice this progression is completely abolished in the E14.5 fetal liver. On the other hand, in Klf1/-Ifnar1/- double-deficient mice, the normal kinetic of fetal liver erythropoiesis is partially re-established with a significant progressive increase of the erythroblastic populations closer to the physiological pattern (Fig. 5).

This result suggests that in absence of Klf1, the impairment of definitive erythropoiesis is not only due to a direct effect of Klf1 on erythropoiesis, but also to a secondary effect due to Interferon-β activation in CMEI. The slower differentiation rate is most likely caused by a partial failure of erythroid specific cell cycle control due to the absence of Klf1 [6, 7, 36]. β-globin imbalance (β-thalassemia) may also contribute to the decreased differentiation of erythroid cells [37].

Notably, in absence of the Interferon type I receptor, Klf1/- orthochromatophilic erythroblasts are able to enucleate and produce reticulocytes, even though to a lesser extent than the WT control. This result indicates that Klf1/- erythroblasts are able to partially overcome the stall at the orthochromatophilic stage [7] and to produce some reticulocytes that enter the blood stream. This block at the orthochromatophilic stage is proposed to be caused by the downregulation of Klf1 cell cycle target genes, p18 and p27 [7]. We have shown that the partial recovery of enucleation in Klf1/-Ifnar1/- double-deficient mice is not linked to the recovery of either p18 or p27 expression (see Supplementary Information). Most likely this result is related to the amelioration of CMEI, and of the associated blood island, which is pivotal for efficient enucleation [10–12]. The improvement in definitive erythropoiesis and enucleation is indeed associated with a partial recovery of the absolute number and shape of macrophages in the Klf1/-Ifnar1/- fetal liver compared to Klf1/-Ifnar1-/+ mice. These data indicate a prolonged survival of the CMEIs and of the associated erythroblastic islands. However, the ratio between stellate and non-stellate macrophages, although higher than that of the single Klf1 KO, is inferior of the WT control mice. These results are in agreement with the notion that Klf1 plays an important direct role in erythroblastic island biology and integrity [38–40].

Our data also suggest that the previously observed perturbation of the apoptotic program [32], is caused, at least in part, by IFN-β activation in Klf1 null fetal liver. The perturbation of the apoptotic program has been suggested to contribute to the failure of terminal erythroid differentiation in Klf1 null mice [32]. Our data support the idea that the lack of Ifnar1, by hampering IFN-β-induced apoptosis, is the main cause of the observed improvement of erythroid differentiation in Klf1/-Ifnar1/- mice. Moreover, we have shown that the observed improvement in definitive erythropoiesis of Klf1/-Ifnar1/- compared to Klf1/-Ifnar1-/+ is not directly due to an increase in the expression of key Klf1 target genes (see Supplementary Information). Unlike the nucleated erythroid cells produced in the yolk sac, those produced in the fetal liver and bone marrow are enucleated. Definitive erythropoiesis in both bone marrow and fetal liver takes place in erythroblastic islands [10, 11]. In Klf1 null mice the primitive embryonic erythropoiesis allows the embryos to survive until the fetal liver definitive erythropoiesis takes place, even though most of the genes affected by Klf1 deprivation are expressed in embryonic as well as definitive erythroid cells [22]. The absence of CMEI, with the consequential lack of IFN-β production in Klf1 null primitive erythropoiesis, may partially explain this difference. Globin imbalance also certainly contributes to
the difference in the level of impairment of erythropoiesis between primitive and definitive erythroid production.

The central role of Klf1 in erythropoiesis is confirmed by our study, which however highlights a contribution of IFN-β that exacerbates the Klf1 null phenotype in mice. The overlap of direct and secondary effects could have given rise to an over-estimation of same aspect of Klf1’s role in erythrocyte development. In this study we have shown that in the absence of Ifnar1, which mediates the cellular response to IFN-β, erythropoiesis and enucleation are significantly improved in the E14.5 Klf1 null fetal liver.

Recent studies have shown that mutations of Ifnar1 are not rare, are under selection, and in some populations may reach appreciable frequencies [20]. Klf1 mutations result in a spectrum of serious diseases up to hydrops fetalis [3], which, although rare, is probably underestimated. In this respect, the knowledge of the pathophysiological mechanisms underlying Klf1 null phenotype may help for an effective counseling and therapeutic approach of these diseases.

In this report, we have shown that the absence of IFN-β cellular response attenuate the Klf1 null phenotype. We hypothesize that a further amelioration of erythropoiesis in Klf1−/−Ifnar1−/− mice would be realized if the globin imbalance were corrected, but this remains to be established. However, if this is indeed the case, Klf1 could be re-evaluated as a target for γ-globin gene reactivation as a therapy for β-thalassemia and sickle cell disease [17–19].

**Material and methods**

**Mice**

The original Klf1 KO (provided by Dr. Frank Grosveld’s laboratory [1]) and Ifnar1 KO (bought from Riken Bio Resource Center, Japan) mouse lines were maintained on a hybrid C57BL/6/CBA/J background. Evaluation of the embryos size has been carried out on at least two pregnancies and four embryos per genotype. Evaluation of fetal liver cellularity has been carried out on at least two pregnancies and three embryos per genotype. Evaluation of the in utero survival has been carried out on at least two pregnancies and three embryos per genotype.

All procedures conducted on the animals were in accordance to the rules and regulations set by the Ethical Committee (OPBA) of University of Cagliari (Approval number: 13/2016).

**Genotyping**

Genotypes were determined by PCR from genomic DNA using a pair of primers in the neomycin-resistant gene (Neo

Fw 5′-ATGGGATCGCCATTGAAC-3′; Neo/Rev 5′-CTCGTCTGCAAGTTCATTC-3′) and a pair of primers in the Klf1 gene (Klf1/Fw 5′-CCACACACATATCGCACA-3′; Klf1/Rev 5′-TGCCGGCTCCCCACACACTC-3′) to discriminate between the WT and KO Klf1 gene.

WT and Ifnar1 KO were detected with a wild-type-specific (5′-AAGATGTGCTGGCAATCTGCTGA-3′) or mutant-specific (5′-CCTGCGTGCAATCCATC TTG-3′) reverse primer and a common forward primer (5′-ATTATAAAAAGAAAGACGGCAGGAGTG-3′).

**Real-time quantitative PCR (RT-qPCR)**

Total RNA was extracted from E14.5 fetal livers using RNeasy Mini Kit (Qiagen) as described by the manufacturer’s protocol. The cDNA was made from total RNA using Superscript III reverse transcriptase (Invitrogen). RT-qPCRs were performed using SYBR Green chemistry (Applied Biosystems) by ABI PRISM 7900 thermocycler (Applied Biosystems, Foster City, CA).

The reactions were performed at least on three different samples in triplicate. See additional data for primers sequence. Samples were normalized with respect to HPRT levels. The analysis of RT-qPCR data was done using the ΔΔCT method.

**Flow cytometry analysis**

Freshly isolated E14.5 fetal liver cells (at least three samples per genotype) were collected and stained with anti-mouse CD44 FITC, and anti-mouse CD45 PE antibodies (BD-Bioscience) at a final concentration 1:100. Cells were incubated for 20 min at 4 °C. Stained cells were then washed with PBS (5% BSA) and resuspended in FACS buffer. Propidium Iodide (BD Pharmingen) were added and incubated for 10 min at room temperature. Data were collected on a FACSCANTO flow cytometer (BD-Bioscience) and analyzed with FACS DIVA software Version 6.1.3 (BD Biosciences) and FloJo V7.6.5.

**Histochromochemical analysis**

The fetal liver samples obtained from E14.5 mice (at least three samples per genotype) were fixed with 4% paraformaldehyde, included in an embedding medium [41] and cut (at 10 m) using a HM-560 cryomicrotome (Microm; Walldorf, Germany). Sections were incubated overnight with rat anti-mouse F4/80 (MCA497R; AbD Serotec, Oxford, UK) monoclonal antibody (1:200) revealed with a secondary antibody conjugated with cyanine 3.18 (1:300, 60 min, Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were observed and photographed using BX51 fluorescence microscope (Olympus, Milan, Italy).
equipped with the Fuji S3 Pro digital camera (Fujifilm, Milan, Italy). Routine controls included substitution of primary antibody with PBS and/or dilution buffer only. Quantitation of macrophages was then carried out on five non-adjacent sections per samples and 4/5 fields per section.

Morphological examination was made in fetal liver sections (at least three samples for genotype) stained with hematoxylin/eosin.

Cytospins were prepared with 5 µl of fetal liver cells suspension (five mice per genotype, three slides per mouse) at a final concentration of 1 × 10^5 cell/µl. Slides were stained with Differential Quik Stain Kit (Modified Giemsa) (Polysciences, Inc.).

Western blotting

Fetal liver E14.5 whole-cell protein extracts were prepared using RIPA buffer and resolved on 4%-12% Bis-Tris precast gels (Invitrogen). Blotting was performed by transferring proteins to PVDF membrane (GE Healthcare), blocking, and incubating with the appropriate primary and a conjugated secondary antibody. The primary antibodies used were as follows: anti-Cleaved Caspase-3 (9661 Cell Signaling Technology) and anti-Actin, (sc-1616 Santa Cruz).

TUNEL (terminal deoxynucleotidyl transferase dUTP nick and labeling) assay

A cell suspension of E14.5 fetal liver cells were assayed for apoptosis using the TUNEL method (three samples per genotype). Cells were marked with anti-mouse CD71 PE (BD-Bioscience) antibody. Cells were prepared according to the manufacturer’s recommendations using the In Situ Cell Death Detection Kit, Fluorescein (Roche). Analysis was carried out using a FACSCANTO flow cytometer (BD-Bioscience) and analyzed with FACSDiva software Version 6.1.3 (BD Biosciences) and FloJo V7.6.5.

Statistical analysis

Statistical differences between means were calculated with Student’s t-test (Microsoft Excel).

Acknowledgements We thank Maria Franca Marongiu, Valeria Faa, and Paolo Moi for kindly reading the manuscript; Daniela Poddie, Alessia Desogus, and Alessia Loi for technical support, Emilio Melis for animal care and technical assistance and Michael Whalen for proofreading the manuscript. Professor Andrea Perra for histopathological examination of the fetal liver.

Authors’ contributions M.F.M., C.B., C.A.C., E.M., M.S, I.A., S.P., and L.P. performed the experiments and contribute to the interpretation of the results. M.F.M., C.B., C.A.C., E.M., M.S., and S.P. contributed to the writing of the manuscript and prepared the figures. M.S.R. and M.F.M. designed the study. M.S.R. designed the study, supervised the research and wrote the manuscript. All authors contributed to the discussion and approved the final manuscript.

Compliance with ethical standards

Competing interests The authors declare that they have no competing financial interests.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License, which permits any non-commercial use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. If you remix, transform, or build upon this article or a part thereof, you must distribute your contributions under the same license as the original. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/4.0/.

References

1. Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature 1995;375:316–8.
2. Perkins AC, Sharpe AH, Orkin SH. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature 1995;375:318–22.
3. Magor GW, et al. KLF1-null neonates display hydrops fetalis and a deranged erythroid transcriptome. Blood 2015;125(15):2405–17.
4. Tallack MR, Perkins AC. KLF1 directly coordinates almost all aspects of terminal erythroid differentiation. IUBMB Life 2010;62(12):886–90.
5. Siatecka M, Bieker JJ. The multifunctional role of EKLF/KLF1 during erythropoiesis. Blood 2011;118:2044–54.
6. Pilon AM, et al. Failure of terminal erythroid differentiation in EKLF deficient mice is associated with cell cycle perturbation and reduced expression of E2F2. Mol Cell Biol 2008;28(24):7394–401.
7. Gnanapragasam MN, McGrath KE, Catherman S, Xue L, Palis J, Bieker JJ. EKLF/KLF1-regulated cell cycle exit is essential for erythroblast enucleation. Blood 2016;12(12):1631–41.
8. Porcu S, et al. KLF1 affects DNase II-alpha expression in the central macrophage of a fetal liver erythroblastic island: a non-cell-autonomous role in definitive erythropoiesis. Mol Cell Biol. 2011;31:4144–54.
9. Bessis M. Erythroblastic island, functional unity of bone marrow. Rev Hematol 1958;13:8–11.
10. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. Blood. 2008;112:470–8.
11. Manwani D, Bieker JJ. The erythroblastic island. Curr Top Dev Biol 2008;82:23–53.
12. de Back DZ, Kostova EB, van Kraaij M, van den Berg TK, van Bruggen R. Of macrophages and red blood cells; a complex love story. Front Physiol 2014;5(5):9.
13. Kawane K, et al. Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver. Science 2001;292:1546–9.
14. Yoshida H, Okabe Y, Kawane K, Fukuyama H, Nagata S. Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. Nat Immunol 2005;6:49–56.
15. Evans CJ, Aguilera RJ. DNase II: genes, enzymes and function. Gene 2003;322:1–15.

16. Kitahara Y, Kawane K, Nagata S. Interferon-induced TRAIL-independent cell death in DNase II-/- embryos. Eur J Immunol 2010;40(9):2590–8.

17. Sankaran VG. Targeting therapeutic strategies for fetal hemoglobin induction. Hematology Am Soc Hematol Educ Program. 2011;2011:459–65.

18. Bauer DE, Kamran SC, Orkin SH. Reawakening fetal hemoglobin: prospects for new therapies for the β-globin disorders. Blood 2012;120(15):2945–53.

19. Zhou D1, Liu K, Sun CW, Pawlik KM, Townes TM. KLF1 regulates BCL11A expression and gamma- to beta-globin gene switching. Nat Genet 2010;42(9):742–4.

20. Perkins A, et al. KLF1 Consensus Workgroup. Krüppel-like factor KLF1. Blood 2016;127(15):1856–62.

21. Müller U, et al. Functional role of type I and type II interferons in antiviral defense. Science 1994;264(5167):1918–21.

22. Hodge D, et al. A global role for EKLF in definitive and primitive erythropoiesis. Blood 2006;107(8):3359–70.

23. Singleton BK, Burton NM, Green C, Brady RL, Anstee DJ. Mutations in EKLF/KLF1 form the molecular basis of the rare blood group In(Lu) phenotype. Blood 2008;112(5):2081–8.

24. Arnaud L, et al. A dominant mutation in the gene encoding the erythroid transcription factor KLF1 causes a congenital dyserythropoietic anemia. Am J Hum Genet 2010;87(5):721–18.

25. Libani IV, et al. Decreased differentiation of erythroid cells exacerbates ineffective erythropoiesis in beta-thalassemia. Blood 2008;112(3):875–85.

26. Xue L, Galdass M, Gnanapragasam MN, Manwani D, Bieker JJ. Extrinsic and intrinsic control by EKLF (KLF1) within a specialized erythroid niche. Development 2014;41(11):2245–54.

27. Liu J, et al. Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. Blood. 2013;121:e43–49.

28. Gordon S, Crocker PR, Morris L, Lee SH, Perry VH, Humke DA. Localization and function of tissue macrophages. Ciba Found Symp 1986;118:54–67.

29. Morris L, Graham CF, Gordon S. Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. Development 1991;112:517–26.

30. Testa U. Apoptotic mechanisms in the control of erythropoiesis. Leukemia 2004;18:1176–99.

31. Sarvothaman S, Undi RB, Pasupuleti SR, Gutti U, Gutti RK. Apoptosis: role in myeloid cell development. Blood Res 2015;50:73–79.

32. Tallack MR, et al. Novel roles for KLF1 in erythropoiesis revealed by mRNA-seq. Genome Res 2012;22(12):2385–98.