Hematological findings in non-treated and γ-irradiated mice deficient for MIC-1/GDF15

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Short title: Hematological analysis in GDF15 knockout mice

Summary

Several members of the TGF-ß family are known to effectively regulate the fate of hematopoietic progenitor cells in a complex and context-dependent manner. Growth differentiation factor-15 (GDF15) is a divergent member of the TGF-ß family. This stress-induced cytokine has been proposed to possess immunomodulatory functions and its high expression is often associated with progression of a variety of pathological conditions. GDF15 is also induced by chemotherapy and irradiation. Very few fundamental studies have been published regarding the effect of GDF15 in hematopoiesis. In this study, we analyzed the hematological status of untreated and γ-irradiated mice deficient for GDF15 as a result of genetic knock-out (KO), in order to clarify the regulatory role of GDF15 in hematopoiesis. Significant differences between GDF15 KO mice and their pertinent WT controls were found in the parameters of blood monocyte numbers, blood platelet size, and distribution width, as well as in the values of bone marrow granulocyte/macrophage progenitor cells. Different tendencies of some hematological parameters in the GDF15 KO mice in normal conditions and those under exposure of the mice to ionizing radiation were registered. These findings are discussed in the context of the GDF15 gene function and its lack under conditions of radiation-induced damage.

Key words: GDF15 knockout mice; hematopoiesis; ionizing radiation; stress-induced cytokine

Introduction

Growth differentiation factor-15 (GDF15, other synonyms MIC-1, NAG-1, PLAB or PTGF-β) is a divergent cytokine belonging to the transforming growth factor-β (TGF-β) family (Bootcov *et al.* 1997). GDF15 pertains to stress-induced cytokines, has been proposed to possess immunomodulatory functions, and its high expression is often associated with cancer progression (Boire *et al.* 2017; Murielle *et al.* 2010), as well as with increased radioresistance of cancer cells (Schilling-Tóth *et al.* 2014). Its production is also associated

with the regulation of appetite and body weight (Tsai *et al.* 2013). Recently, the orphan receptor GFRAL has been identified as a mediator for GDF15-mediated metabolic effects (Emmerson *et al.* 2017; Mullican *et al.* 2017; Yang *et al.* 2017).

Very few fundamental studies have been published regarding the effect of GDF15 in hematopoiesis. GDF15 was originally described as macrophage inhibitory cytokine 1 (MIC-1) (Bootcov*, et al.* 1997). Subsequent studies have discovered an association of GDF15 with both inhibition and activation of macrophages (Fairlie *et al.* 1999), suggesting context-specific effects of this cytokine. High levels of GDF15 expression have been also detected in cultured erythroblasts reflecting ineffective erythropoiesis (Tanno *et al.* 2010). Notably, GDF15 has been proposed as a hepcidin-suppressing factor that is expressed at high levels in patients with ineffective erythropoiesis (primarily in β-thalassemia) and iron overload (Tanno *et al.* 2007). Hepcidin is a principal regulator of iron absorption and its tissue distribution (Ganz *et al.* 2011). It also inhibits iron release from macrophages and iron uptake by intestinal epithelial cells (Davis *et al.* 2012), thus it can be considered an erythropoiesis-suppressing or anemia-producing factor.

Concerning hematological parameters of white blood cells, GDF15 has been identified as an inhibitor of polymorphonuclear leukocyte recruitment by direct interference with chemokine signaling and integrin activation; loss of this anti-inflammatory mechanism leads to fatal cardiac rupture after myocardial infarction (Kempf *et al.* 2011). An immunoregulatory (immunosuppressive) function of recombinant GDF15 has been described also in our laboratory (Soucek *et al.* 2010). These fragmentarily described hematological effects of GDF15 and their contexts suggest that GDF15 hypothetically possesses abilities to influence both the white cell and the red cell hematopoietic compartments. However, a complex hematological study, which would shed light on contingent hematopoiesis-modulating capabilities of GDF15, is still missing.

GDF15 deficient mice represent a suitable methodological approach for this task. The loss of GDF15 activity is chronic (lifelong) and absolute. Here we performed a complex analysis of hematopoiesis in non-treated and γ-irradiated GDF15-deficient (GDF15 knock-out, GDF15 KO) mice. We hypothesized that the mice with the missing GDF15 gene should

show an increased production of hepcidin and, thus, a suppressed erythropoiesis. Concomitantly, the lack of the GDF15 gene which is responsible for a macrophage inhibitory function prompted us to investigate the changes in the production of monocytes/macrophages observable at the levels of the bone marrow hematopoietic progenitor cells for granulocytes/monocytes (GM-CFC) and peripheral blood monocytes. The experimental exposure of the mice to ionizing radiation has been chosen since it stands for a well-defined stress and detrimental approach affecting all hematopoietic compartments. Our findings show that the loss of the GDF15 gene does not significantly affect erythropoiesis, whereas it has rather supportive effects on some leukopoietic compartments.

Material and Methods

Mice

Founders GDF15$^{-/-}$ mice on the C57BL/6 genetic background (Hsiao *et al.* 2000) were obtained from the laboratory of Prof. Se-Jin Lee, Johns Hopkins University School of Medicine, Department of Molecular Biology and Genetics (Baltimore, MA, USA). Animals used for subsequent analyses were generated from breeding described in the scheme below at the animal facility of the Veterinary Research Institute, Brno, Czech Republic; median age of the animals used in the study was 16 weeks. Average body weight in untreated WT and GDF15 KO mice was 23.8 g and 25.3 g, respectively, that in irradiated WT and GDF15 KO mice was 23.9 g and 24.1 g, respectively.

Colony 1: C57BL/6 GDF15$^{+/-}$ males x C57BL/6 GDF15$^{+/-}$ females

Resulting offspring: **25% GDF15$^{-/-}$ mice**, **25% GDF15$^{+/+}$ mice**, **50% GDF15$^{+/-}$ mice**

GDF15$^{+/-}$ mice were used as replacement breeders for Colony 1, GDF15$^{-/-}$ mice were used as breeders for Colony 2, GDF15$^{+/+}$ mice were used as breeders for Colony 3.

Colony 2: C57BL/6 GDF15$^{-/-}$ males x C57BL/6 GDF15$^{-/-}$ females

Resulting offspring**: 100% GDF15$^{-/-}$ mice (experimental GDF15-deficient mice).**

Colony 3: C57BL/6 GDF15$^{+/+}$ males x C57BL/6 GDF15$^{+/+}$ females

Resulting offspring**: 100% of GDF15$^{+/+}$ mice (experimental WT controls).**

This approach to <u>obtain</u> experimental GDF15-deficient mice and WT controls guaranteed that the two groups of mice differed solely in the expression of the GDF15 gene. The GDF15 genetic status of all mice was determined by routine PCR genotyping. Isolation of genomic DNA from mouse ears and subsequent PCR was performed with REDExtract-N-Amp™ Tissue PCR Kit (cat. n. XNAT, Sigma-Aldrich). Mice were genotyped using specific sets of primers for WT and KO alleles (WT-Fw, 5'- CCT GGA GAC TGT GCA GGC AAC TCT TG -3'; WT-Re, 5'- GTG ACA CAC CAC TGT CTG TCC TGT GC -3'; KO-Fw, 5'- GCT GTC CGG ATA CTC AGT CCA GAG G -3'; KO-Re, 5'- CGC CTT CTT GAC GAG TTC TTC TGA GGG -3'). Amplification was carried out under following conditions (94 °C, 30 s; 68 °C, 20 s; 72 °C, 30 s; 35 cycles). The expected size of PCR products for WT or KO alleles was 228 and 598 bp, respectively (not shown).

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted, including the EU Directive 2010/63/EU for animal experiments.

Irradiation

The mice were whole-body irradiated at a dose rate of 0.33 Gy/min using a γ-ray source (^{60}Co, Chisostat, Chirana, Prague, Czech Republic). A single sublethal dose of 4.0 Gy was used.

Hematological techniques

For evaluation of the peripheral blood parameters, the animals were anesthetized 72 h after irradiation with an i.p. injection of 0.07 ml of Narcamon/Rometar solution in a ratio of 2.63:1 (5% Narcamon, Spofa, Prague, Czech Republic and 2% Rometar, Bioveta, Ivanovice na Hané, Czech Republic), and the peripheral blood was sampled by cardiac puncture. The

numbers of total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, erythrocytes, and platelets per 1 µl of the peripheral blood were determined by an Auto Hematology Analyzer Mindray 5300Vet (Shenzhen Mindray Bio-Medical Electronics Co, Shenzhen, China). This analyzer allows us to determine the numbers of leukocyte subtypes per 1 l of the peripheral blood only when the total leukocyte number exceeds the threshold of 0.5 x 10^9/l and, therefore, these parameters could not be determined in the irradiated mice. The same device was used for determination of blood hemoglobin level (HGB), hematocrit (HCT), mean erythrocyte volume (MCV), mean erythrocyte hemoglobin (MCH), mean erythrocyte hemoglobin concentration (MCHC), red blood cell distribution width (RDW), mean platelet volume (MPV), plateletcrit (PCT), and platelet distribution width (PDW).

Femurs were removed from mice sacrificed by cervical dislocation, marrow cells were harvested by standard procedures, and the numbers of nucleated cells of the femoral bone marrow were determined using a Coulter Counter (Model ZF, Coulter Electronics, Luton, UK). Standard procedures were used for the *in vitro* assays of the femoral clonogenic progenitor cells. Granulocyte-macrophage colony-forming cells (GM-CFC) were assessed using the MethoCult GF M3534 medium (StemCell Technologies, Vancouver, Canada). Erythroid progenitor cells (BFU-E) were determined using the MethoCult M3334 medium (StemCell Technologies). Femoral marrow cell suspensions were plated (1.5 x 10^5 and 1 x 10^5 nucleated bone marrow cells for GM-CFC and BFU-E, respectively) in triplicate for both assays and incubated at 37° C in a humidified atmosphere containing 95% air and 5% CO_2. GM-CFC were scored after a 7-day incubation as colonies containing 50 or more cells. Hemoglobinized colonies were counted as BFU-E after an 8-day incubation. The values of the femoral GM-CFC and femoral BFU-E were expressed as per femur and as per 10^5 femoral bone marrow cells. Taking into account the body weight differences between the GDF15 KO mice and their WT counterparts, the values of femoral bone marrow cellularity, femoral GM-CFC, and femoral BFU-E were expressed also as per g body weight.

Flow cytometric analysis

Bone marrow cells and blood were collected as described above. The spleen was dissected, minced to 1-2 mm pieces and mechanically dissociated with GentleMACS Dissociator

(program m_spleen_01; Miltenyi, Bergish Gladbach, Germany). 100 μL of full blood or approximately $1x10^6$ bone marrow cells or splenocytes were washed with PBS, blocked with 2% normal rat serum (Invitrogen, Thermo Fisher Scientific, Prague, Czech Republic) in PBS to prevent non-specific rat antibody binding for 10 min at ambient temperature. After blocking, cell suspensions were stained with the CD11b-APC antibody (dilution 1:20, cat. RM2805, clone M1/70.15, Thermo Fisher Scientific), and then with viability stain (LIVE/DEAD Yellow Cell Stain Kit, 1:500; Molecular Probes, Thermo Fisher Scientific) for 15 min at ambient temperature. Red blood cells were lysed with ACK buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate and 100 μM EDTA solution in sterile MQ water) for 5 min at 37°C. Samples were then re-suspended in PBS and analyzed. Data acquisition was performed with FACSVerse (Becton Dickinson Biosciences, Franklin Lakes, New Jersey, United States). Acquired FCS files were exported and analyzed using FlowJo Software (v10.0.7; TreeStar, Ashland, Oregon, United States). Cell aggregates and debris were excluded from analysis based on a dual-parameter dot plot in which the pulse ratio (signal height/y-axis versus signal area/x-axis) was displayed. Dead cells were excluded from analysis based on LIVE/DEAD Yellow stain positivity. Spectral compensations were calculated automatically in data acquisition software using single conjugate stained UltraComp eBeads and verified using fluorescence-minus-one controls (eBioscience, Thermo Fisher Scientific).

Histology

For tissue harvesting, mice were euthanized as described above. Spleen and both femurs, cleaned from surrounding muscles, were dissected and directly fixed in 10% neutral buffered formalin solution overnight. Femurs were then washed in tap water and decalcified (4% HNO_3, 0.15% CrO_3). Tissues were dehydrated and embedded in paraffin using routine procedures. First, formalin-fixed, paraffin-embedded (FFPE) tissue blocks were cut to 4-μm-thick tissue sections and deparaffinized by xylene wash and rehydrated in concentrations of ethanol. Sections were then counterstained with Mayer's hematoxylin and eosin, dehydrated, cleared by xylene and mounted using the Pertex medium. Slides were scanned using TissueFAXS system (TissueGnostics).

Data mining and bioinformatic analysis

Promoter, regulatory and encoding regions of mouse and human GDF15 gene were obtained from Ensembl (http://ensembl.org, (Consortium 2008; Yates *et al.* 2015)) or NCBI databases (http://ncbi.nlm.nih.gov, (Benson *et al.* 2005)) and mutually compared at various levels using multiple web-based tools; first, genomic DNA (5´UTR/3´UTR 1500 bp upstream or downstream and coding regions), corresponding mRNA (NM_004864.2; NM_011819) and protein (Q99888.3; Q9Z0J7) sequences were all aligned using BLAT/BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi; (Altschul *et al.* 1990)). Analyses of conserved transcription factors (TFs) binding sites (TFBSs) were performed and compared between corresponding regions of mouse and human GDF15 (1500up 5´TSS and first exon) using rVista (http://rvista.dcode.org/; (Loots *et al.* 2004)) alignment tool. *In silico* analysis of TFBSs prediction were all performed in Genomatix software using MatInspector, ModelInspector and Overrepresented transcription factor binding sites/modules tools (Z score cut off < -2 or >2; (Cartharius *et al.* 2005; Ho Sui *et al.* 2005)), and compared with results obtained from Encode database (https://www.encodeproject.org; (2012)). Expression values (logFC) of microarray probes mapping to mouse and human GDF15 genes were obtained from various experiments deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) or ArrayExpress databases (https://www.ebi.ac.uk/arrayexpress/) and analysed using Geo2R (Davis *et al.* 2007) or BRB Array tools (http://brb.nci.nih.gov/BRB-ArrayTools/index.html; (Simon *et al.* 2007)) (data not shown).

Statistical methods

The values are presented as arithmetic means ± standard errors of the means (SEM). In each parameter, the global statistical significance among the groups was assessed using one-way ANOVA. The differences between individual experimental groups were evaluated using the Mann-Whitney test.

Results

The basis for this experimental study originated in comparative analysis of available genomic and microarray data for human and mouse GDF15 gene. Although genes encoding human and mouse GDF15 are located on different chromosomal strands in opposite directions, they share similar neighboring genes (Ensembl; http://ensembl.org). Yet, alignment of the promoter and large parts of raw encoding regions did not yield any similarity (BLAST/BLAT; rVista; data not shown), although GDF15 mRNA and protein sequences share ~70% and ~62% similarity between human and mouse, respectively.

At first, we focused our attention on scanning through publicly available microarray databases (Gene Expression Omnibus- GEO and Array Express) to address the assumption, that mouse GDF15 gene expression responds to ionizing radiation by its accelerated induction, as was shown for the human homologue (Sándor *et al.* 2015). Indeed, analyses of previously published microarray datasets obtained from control mice and their counterparts exposed to various doses of ionizing radiation revealed statistically significant (data not shown) induction of mGDF15 expression in various hematopoietic tissue samples (peripheral blood, bone marrow). Considering this scanning through microarray databases, we further performed *in silico* analyses to uncover transcription factors, which may regulate GDF15 expression in both tested species and are associated with hematopoiesis and irradiation stress (Genomatix software suite and Encode database). As expected, binding sites for p53, NFkB, E2F1 and other TFs associated with cellular response to radiation stress were computationally predicted, significantly overrepresented and some of them have been already confirmed to bind DNA regions in GDF15 encoding locus (Table 1) (2012; Malewicz *et al.* 2014; Osada *et al.* 2007). Interestingly, analysis of putative binding sites specific for TFs engaged during various stages of hematopoiesis revealed that some of them are either directly occupied (e.g. CEBPβ, GATA1, GATA2, SCL/TAL1 and Myc), or are at least predicted/overrepresented (e.g. GFI1, Ikaros, JunB, Hif1a, Smad1/5) in GDF15 regulatory regions of both human and mouse origin (Table 2); (Hattangadi *et al.* 2011; Rosenbauer *et al.* 2007)). Finally, the relevance of this study is supported by the knowledge that GDF15 expression in mice occurs i.e. in hematopoietic organs and tissues such as foetal, neonate and juvenile liver, in adult spleen and thymus, and in placenta [Gene expression database

(http://www.informatics.jax.org/expression.shtml, (Smith *et al.* 2014)) and Gene expression atlas (https://www.ebi.ac.uk/gxa/home; (Kapushesky *et al.* 2010))]. These findings thus clearly indicate a complex association of regulatory mechanisms driving GDF15 expression with distinct stages of hematopoiesis.

Therefore, we aimed to investigate whether the loss of GDF15 expression in the mouse affects hematopoiesis in both genders of untreated mice and in those exposed to a sublethal dose of ^{60}Co γ-rays.

Our analysis of peripheral blood leukocyte parameters revealed a significant decrease of the numbers of blood monocytes in untreated GDF15 KO mice, both males and females (Fig. 1B). Because of very low values of total blood leukocytes in irradiated animals (Fig. 1A), lying below the threshold of the analyzer used (0.5×10^9/l), this parameter was impossible to determine in radiation-exposed mice.

Parameters of the peripheral blood erythrocytes (Fig. 1C,D) were not outstandingly different between GDF15 KO mice and their WT counterparts, either in untreated or in irradiated animals. The only exceptions were a significant elevation of the red cell distribution width (RDW) in irradiated GDF15 KO males and a small but significant decrease of the mean erythrocyte hemoglobin (MCH) value in untreated GDF15 KO females. Given the long life-span of blood erythrocytes, further studies are needed to reveal contingent effects of the lack of the GDF15 gene on blood erythrocyte parameters.

Among the peripheral blood platelet parameters, the most prominent finding is a significantly increased value of the mean platelet volume in irradiated GDF15 KO males (Fig. 1E-G). Since the platelet volume was higher also in irradiated female GDF15 KO mice than in their WT counterparts, though without achieving statistical significance, this comparison reaches statistical significance also when both sexes were evaluated together (not shown). A moderate but statistically significant increase in the parameter of the platelet distribution width (PDW) in favor of the irradiated GDF15 KO male mice when compared to their WT counterparts was also found (Fig. 1F); due to a similar, though statistically insignificant difference in this parameter in the irradiated female mice, a significant difference was found for PDW also when both sexes were merged (not shown).

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Findings in the parameters of the bone marrow (Fig. 2A-C) revealed different trends of these parameters between males and females. In untreated males, the femoral bone marrow cellularity expressed as per gram body weight was found to be statistically significantly lower in the GDF15 KO mice than in their corresponding WT counterparts (Fig. 2A); the same was true for this parameter and for the parameter of numbers of GM-CFC if they were expressed as per 10^5 bone marrow cells (not shown). On the contrary, in the irradiated males, the values of marrow GM-CFC and those of marrow hematopoietic progenitor cells for erythrocytes (BFU-E) were observed in all the three ways of expression to be significantly higher in the GDF15 KO mice (for GM-CFC and BFU-E per 10^5 bone marrow cells, see Figs. 2B,C, values of GM-CFC and BFU-E per femur and per femur/g body weight not shown). In the untreated females, the absolute numbers of BFU-E per femur were found to be significantly higher in the GDF15 KO mice. In the irradiated females, the values of GM-CFC per femur and GM-CFC per 10^5 femoral bone marrow cells were significantly higher in the GDF15 KO animals; in contrast to the irradiated males, no significant differences in the values of BFU-E in the femur were found between the GDF15 KO mice and their WT counterparts in the females (for GM-CFC and BFU-E per 10^5 bone marrow cells, see Figs. 2B,C, values of GM-CFC and BFU-E per femur and per femur/g body weight not shown). Noteworthily, both in the irradiated female and irradiated male, bone marrow cellularities were increased in the GDF15 KO mice, and thus, this difference achieved statistical significance ($P \leq 0.05$) when both sexes were merged (not shown). Similarly, when the sexes were merged, the differences in the values of femoral GM-CFC reached statistical significance regardless of the way of the expression (not shown).

To confirm our findings on peripheral blood monocytes and reveal the site of their release, we introduced flow cytometric protocol for analysis of CD11b$^+$ cells in peripheral blood, spleen and femoral bone marrow, where CD11b served as a marker of monocytes and macrophages. As expected, the proportion of CD11b$^+$ leukocytes (CD45$^+$) was decreased in peripheral blood of GDF15 KO males (Fig. 2D). However, in the bone marrow, a significantly higher representation of CD11b$^+$ cells was observed in the GDF15 KO males compared to the WT males (Fig. 2E). In the spleen, no significant differences between GDF15 KO and WT mice were found (Fig. 2F). The further histological analysis did not reveal any

obvious difference in the phenotype of spleen and bone marrow between GDF15 KO mice and their WT counterparts (Fig. 3).

Discussion

Several members of the TGF-ß family, including TGF-ß, bone morphogenetic proteins, and activins are known to effectively regulate the fate of hematopoietic progenitor cells in a complex and context-dependent manner (Söderberg *et al.* 2009). Surprisingly, very few fundamental studies have been published regarding the effect of GDF15 in hematopoiesis. Therefore, in this study, we took advantage of existing GDF15 KO mice and investigated whether the loss of GDF15 expression in this experimental model affects hematopoiesis in untreated and γ-rays irradiated mice. We have discovered significant differences in hematopoiesis in both non-treated and irradiated animals between GDF15 KO mice and their WT counterparts, especially in the leukocytic parameters of the bone marrow and the peripheral blood.

The results presented here show that in the GDF15 KO mice, several hematological parameters are modified in comparison with their WT counterparts, especially those of the peripheral blood monocytes and the bone marrow hematopoietic progenitor cells. The differences between the GDF 15 KO and WT mice include also those in their response to the acute radiation stress.

When the results of our experiments are interpreted in the context of the tasks and hypotheses presented in the Introduction (supposed influence of GDF15 gene loss on erythropoiesis, leukopoiesis, and effects of irradiation on hematopoiesis in GDF15 KO mice), several conclusions and considerations can be made.

First, no important differences in erythropoietic parameters of the peripheral blood between the GDF15 KO mice and the control WT mice were observed. As mentioned in the Introduction, GDF15 should play a suppressive role in the production of an anemia-causing factor hepcidin (Davis*, et al.* 2012; Tanno*, et al.* 2007). However, our results do not confirm any negative impact of GDF15 loss on erythropoiesis. Significantly higher values of the mean

platelet volume (MPV) and the platelet distribution width (PDW) in the irradiated GDF15 KO mice compared to their WT controls (see Fig. 1F,G) can reflect an increase in the intensity of platelet metabolism (valid for MPV, (Park *et al.* 2002)) and of active platelet release (valid for PDW, (Kasperska-Zając *et al.* 2014)) caused by irradiation in these mice. However, the mechanism underlying these differences and their relation to the functions of the GDF15 gene remains unknown at present.

Second, clear signs of changes in the parameters of the peripheral blood monocytes and the bone marrow hematopoietic progenitor cells for granulocytes/monocytes (GM-CFC) were found in the GDF15 KO mice. In untreated GDF15 KO mice, the numbers of blood monocytes were observed to be statistically significantly lower compared to their WT counterparts in both males and females (see Fig. 1B), which held true also for the numbers of the bone marrow GM-CFC in males. On the other hand, in irradiated GDF15 KO mice, the numbers of the bone marrow GM-CFC were found to be significantly higher in both sexes when compared to their WT controls. In our study we did not assess the activity of macrophages (monocyte descendants) whose decrease evoked by GDF15 led to the first denomination of GDF15 as macrophage inhibitory cytokine (MIC-1) (Bootcov, *et al.* 1997). However, we suppose that if GDF15 (or its absence) influenced, in any way, the activity of macrophages, the situation would be reflected in the organism by changes in monocyte production. This confirms the regulatory role of GDF15 in the production of monocytes/macrophages.

Third, the finding of clearly and significantly decreased values of blood monocytes in normal (untreated) GDF15 KO mice by about 40 % in both males and females (see Fig. 1B) raises the question of a potential relationship between this finding and the anticipated absence of the inhibitory role of the GDF15 cytokine towards macrophage activity in GDF15-lacking mice. However, this question cannot be fully answered without further studies aimed at evaluation of macrophage activity in GDF15 KO mice. Nevertheless, it should be stated that the physiological significance of the decreased blood monocyte values in untreated GDF15 KO mice is questionable because normal blood monocyte values in the mouse fluctuate around 0.2×10^9/l (Sanderson *et al.* 1981), which is just the value found in our GDF15 KO mice. Two independent methodical approaches (hematological analyzer, flow

cytometry) confirmed the decrease of monocytic cells in the peripheral blood of the GDF15 KO mice (Figs. 1B and 2D). Interestingly, an opposite trend, i.e. an increased representation of CD11b$^+$ cells in the GDF15 KO mice, at least in the case males, was observed when analyzing their bone marrow (Fig. 2E). This difference in behavior of the monocyte/macrophage cells in the peripheral blood and the bone marrow of the GDF15 and WT mice can be caused by changes in the kinetics of their maturation and release into the peripheral blood or changes in their half-life resulting from the loss of GDF15 gene. This consideration is partially supported by published results, which describe GDF15 as a molecule interfering with CCR2-dependent chemotaxis (de Jager *et al.* 2011), a biological process typical for a subpopulation of monocytes during their emigration from bone marrow into the blood (Shi *et al.* 2011). Recently it has been demonstrated that extravasation of myeloid cells is counteracted by GDF15 through inhibition of integrin activation via canonical TGF-β receptor heterodimers (Artz *et al.* 2016). Loss of this anti-inflammatory mechanism leads e.g. to the fatal cardiac rupture after myocardial infarction (Kempf*, et al.* 2011) which demonstrates prominent role for GDF15 in inflammation (for review see (Breit *et al.* 2016)). However, further studies are needed to elucidate the mechanisms involved in the regulation of cell numbers of the monocyte/macrophage lineage in the peripheral blood and hematopoietic organs in the GDF15 KO mice.

Similar considerations can apply to the increased values of the bone marrow progenitor GM-CFC, appearing only in irradiated GDF15 KO mice (see Fig. 2B). Hypothetically, it could be stated that under exposure to radiation, the anticipated removal of the macrophage inhibitory function of the GDF15 cytokine comes hand in hand with the stimulation of the production of monocytes/macrophages. Of interest is a comparison of our hematological findings concerning the effects of GDF15 and those regarding the action of the bone morphogenetic protein termed PLAB, which is, however, a synonym for GDF15 (Hromas *et al.* 1997). In the latter study, PLAB (GDF15) was reported to inhibit a variety of human hematopoietic progenitor cells including GM-CFC and BFU-E. Considering the findings in both studies, several remarks regarding the role of sex, physiological status, and possible differences between the actions of GDF15 in humans and mice can be made.It follows from these considerations that there exist human vs. mouse, as well as male vs.

female differences in the effects of GDF15 on hematopoiesis. In irradiated mice, we found the values of GM-CFC and BFU-E being always (both in the males and females) higher in GDF15 KO animals than in their WT counterparts, the differences in GM-CFC progenitor cells being significant in the males. This result means that after radiation-induced damage to mouse hematopoiesis, the assumption that the removal of GDF15 plays a positive hematological role is confirmed.

Several parameters evaluated in this study show sex differences, namely those of platelets, bone marrow cellularity, hematopoietic progenitor cells, and CD11b$^+$ cells (see Figs. 1 F,G, 2A,C,D,E). Sex differences are known as distinct responses of males and females to irradiation (e.g., (Kato *et al.* 2013; Koturbash *et al.* 2008)), as well as gender differences observed specifically between GDF15 KO mice and their WT counterparts (Low *et al.* 2017; Zhang *et al.* 2017); they can be also of practical importance in the clinical medicine.

In summary, we have found that there exist significant differences in hematopoiesis in both non-treated and irradiated animals between GDF15 KO mice and their WT counterparts, especially in the leukocytic parameters of the bone marrow and the peripheral blood. Further investigation is needed to assess the mechanisms of GDF15 action in leukopoiesis, and to understand in detail the role of this cytokine in the bone marrow microenvironment under both normal and stressed conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' Contributions

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Tables

Common overrepresented TFs binding sites for human and mouse GDF15			
AP4	HELT	NME/NM23	SP4
CARF	HEN1	NRF1	THR
CTCF	HIF	Nrsf/REST	USF1/2
Dec1/Bhlhe40	KLF3	p53	VDR/RXR
E2F1	KLF5	Pax5	ZBED4
E2F1-DP2	KLF6	PLAG1	Zbp89
E2F7	KLF7	PPARalpha/RXR	ZBTB7
Egr1	Myc	PPARgamma	Zfp67
ERalpha	Max	PRDM5	ZIC2
GC-box elements	MyoD	RAR/RXR	ZIC3
GCM1	NFkB	SP1	ZNF263

Table 1. *In silico* analysis of transcription factor binding sites overrepresentation in human and mouse GDF15 regulatory regions (Genomatix Software Suite- see Materials and Methods). Only hits common for both species and with Z-score values <-2 and >2 are

shown. Blue color depicts TFs confirmed to bind human GDF15 DNA regions located 500 bp upstream or downstream of transcription start site (TSS)(Encode database). Green color depicts TFs confirmed to bind mouse GDF15 DNA regions located 500 bp upstream or downstream of TSS (Encode database). Red color depicts TFs confirmed to bind GDF15 DNA regions specified above in both species (Encode database).

Table 2.

Z-score (genome) < -2 or > 2		MatInspector/OTFBS	
TF name	TF Individual matrices	hGDF15	mGDF15
CEBPa	V$CEBPA.01	+/-	-/-
	V$CEBP.02	-/-	-/-
CEBPb	V$CEBPB.01	+/-	-/-
	V$CEBPB.02	+/-	-/-
CEBPe	V$CEBPE.01	-/-	-/-
	V$CEBPE.02	-/-	-/-
cMYB	V$CMYB.01	-/-	+/-
	V$CMYB.02	-/-	-/-
GATA1	V$GATA1.01	-/-	-/-
	V$GATA1.02	-/-	-/-
	V$GATA1.03	-/-	+/-
	V$GATA1.04	-/-	-/-
	V$GATA1.05	-/-	-/-
	V$GATA1.06	+/-	-/-
	V$GATA1.07	-/-	-/-
GATA2	V$GATA2.01	-/-	-/-
	V$GATA2.02	-/-	-/-
	V$GATA2.03	-/-	-/-
GFI1	V$GFI1.01	-/-	-/-
	V$GFI1.02	-/-	+/-
	V$GFI1B.01	+/-	-/-
HIF1a	V$HIF1.01	+/-	-/-
	V$HIF1.02	+/-	-/-
	V$HRE.01	-/+	-/+
	V$HRE.02	+/+	+/+
	V$HRE.03	-/-	+/-

IRF8	V$IRF8.01	-/-	-/-
Ikaros	V$IK1.01	+/-	+/+
	V$LYF1.01	-/-	-/-
JunB	V$JUNB.01	+/-	+/+
KLF1	V$EKLF.01	-/-	+/+
	V$EKLF.02	-/-	-/+
Myc	V$CMYC.01	-/-	-/-
	V$CMYC.02	+/+	+/+
	V$MYCMAX.01	+/+	-/-
	V$MYCMAX.02	-/-	+/-
	V$MYCMAX.03	-/-	-/-
PU.1	V$SPI1.01	-/-	-/-
	V$SPI1.02	+/-	-/-
	V$SPI1.03	-/-	-/-
	V$SPI1.04	+/-	-/-
	V$SPI1.05	-/-	-/-
RUNX1	V$AML1.01	+/-	-/-
	V$AML1.02	-/-	-/-
SCL/TAL1	V$TAL1ALPHAE47.01	+/+	-/-
	V$TAL1BETAE47.01	+/+	-/-
	V$TAL1BETAHEB.01	+/+	-/-
	V$TAL1BETAITF2.01	-/-	-/-
	V$TAL1_E2A.01	+/-	-/-
	V$TAL1_E2A.02	+/-	+/-
SMAD1/5	V$GC_SBE.01	+/-	+/-

(Genomatix match)	Genomatix match
(Encode match)	Encode match
+/-	TFBSs predicted (MatInspector)
+/+	TFBSs predicted (MatInspector) and significantly overrepresented (OTFBS)
-/-	TFBSs neither predicted nor significantly overrepresented

Table 2. *In silico* **analysis of hematopoietic TF binding sites prediction (MatInspector), overrepresentation (OTFBS)(Genomatix) and direct occupation (Encode) in GDF15 regulatory regions of human and mouse origin**

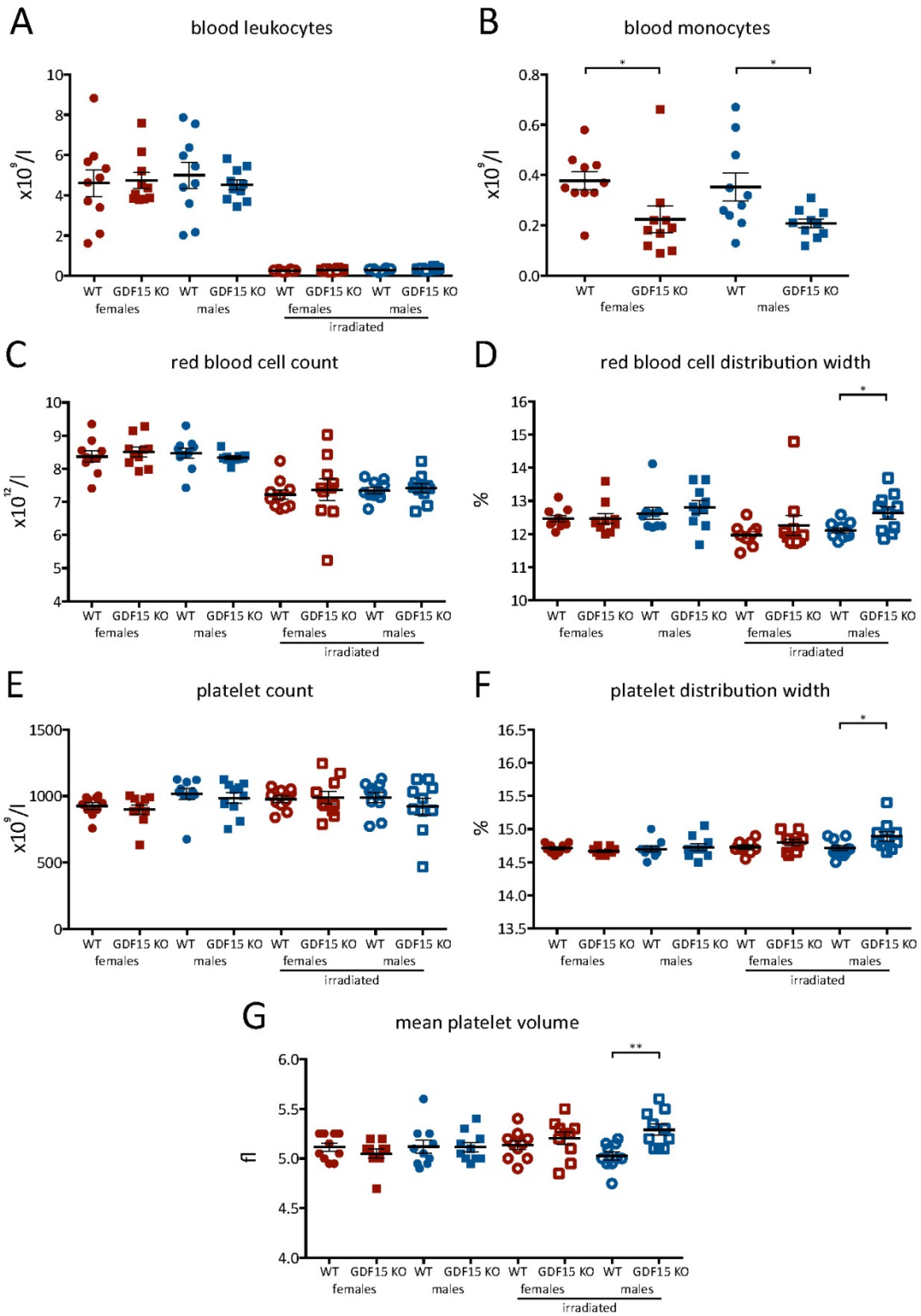**Figures and Legends**

Figure 1

Figure 1. Peripheral blood leukocytes, monocytes erythrocytes and platelets. Scatter dot plots show number of blood leukocytes (A), monocytes (B), red blood cells (RBC) (C), RBC distribution width (D), number of platelets (E), platelet distribution width (F) and mean platelet volume (G) in untreated wild-type (WT), untreated GDF15-deficient (GDF15 KO), irradiated WT, and irradiated GDF15 KO mice. The results are presented as means ± SEM, n = 10 per group. *- P≤ 0.05, ** - P ≤ 0.01, Mann-Whitney test.

Figure 2

A — femoral bone marrow cellularity

B — GM-CFC

C — BFU-E

D — CD11b⁺ cells in blood

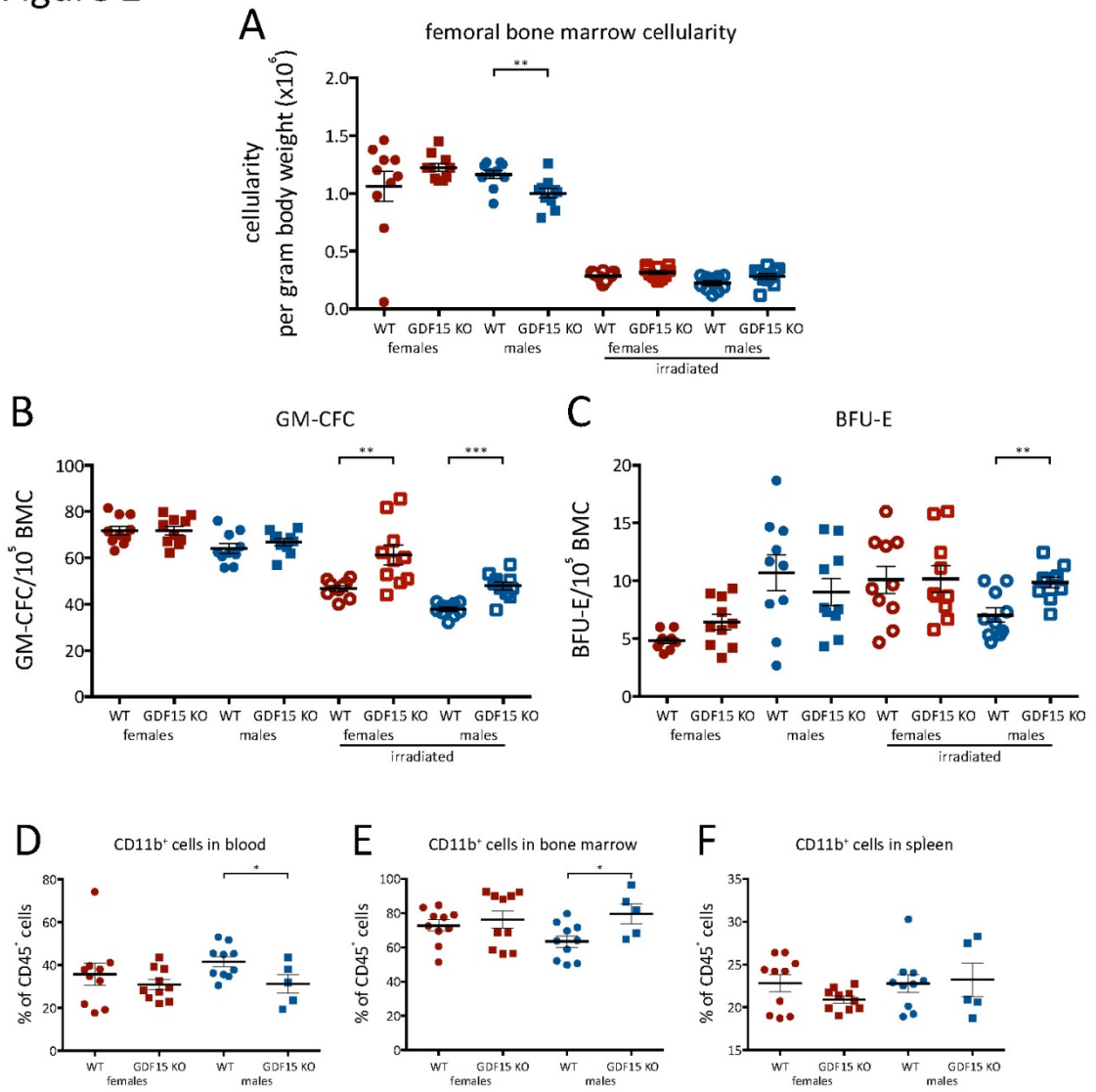E — CD11b⁺ cells in bone marrow

F — CD11b⁺ cells in spleen

Figure 2. Monocyte and erythrocyte progenitors. Scatter dot plots show femoral bone marrow cellularity (A), number of GM-CFC per 10^5 bone marrow cells (BMC; B) and number of BFU-E per 10^5 BMC (C) in untreated WT, untreated GDF15 KO, irradiated WT, and irradiated GDF15 KO mice. The results are presented as means ± SEM, n = 10 per group. *- P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, Mann-Whitney test. **CD11b cells in peripheral blood, bone marrow, and spleen.** Scatter dot plots show the percentage of CD11b-positive leukocytes (CD45$^+$ cells) in the blood (D), bone marrow (E) and spleen (F) in WT and GDF15 KO mice. The results are presented as means ± SEM, n = 10 per all groups except GDF15 KO males, where n = 5. *- P ≤ 0.05, Mann-Whitney test.

Figure 3

A

WT female

GDF15 KO female

WT male

GDF15 KO male

B

WT female

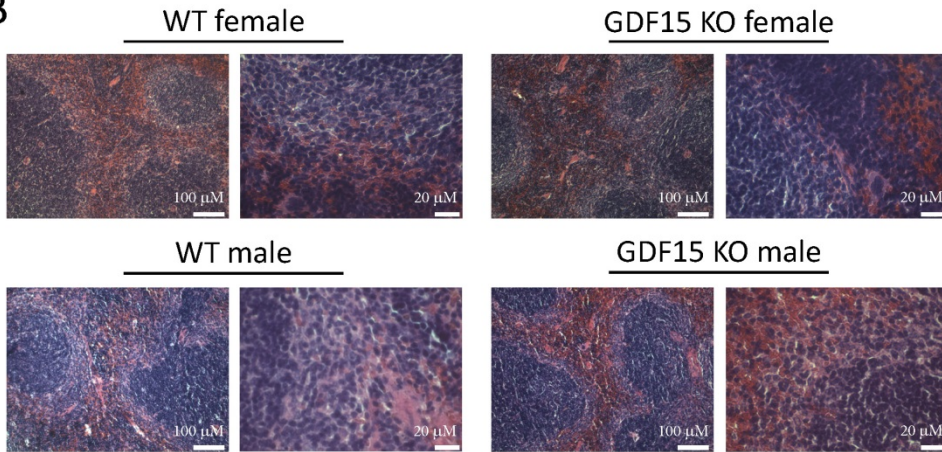GDF15 KO female

WT male
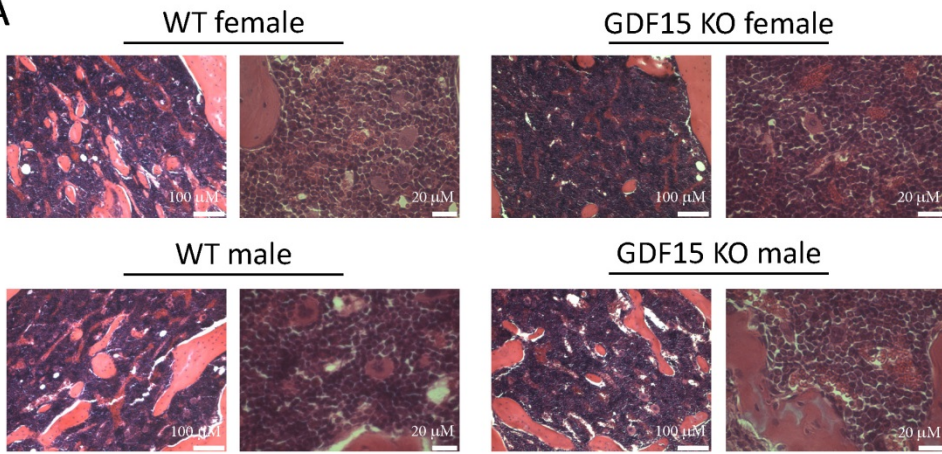
GDF15 KO male

Figure 3. Representative histology of bone marrow (A) and spleen (B) of WT and GDF15 KO female and male mice.