Erythroid cells generated in the absence of specific β1-integrin heterodimers accumulate reactive oxygen species at homeostasis and are unable to mount effective antioxidant defenses

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ABSTRACT

We have previously reported that $\beta1^{\Delta/\Delta}$ mice have a markedly impaired response to hemolytic stress, but the mechanisms of this were unclear. In the present study we explored in detail quantitative, phenotypic and functional aspects of erythropoiesis at homeostasis in a large number of animals for each of 3 murine models with specific β1 heterodimer integrin deficiencies. We found that, at homeostasis, β1-deficient mice have a modest uncompensated anemia with ineffective erythropoiesis and decreased red blood cell survival. Mice lacking only α4 integrins (α4β1/α4β7) do not share this phenotype. There is an increased tendency for reactive oxygen species accumulation in $\beta1^{\Delta/\Delta}$ erythroid cells with decreased anti-oxidant defenses at homeostasis which are exaggerated after stress. Furthermore, expansion of erythroid cells in spleen post-stress is dependent on α5β1, likely through mechanisms activating focal adhesion kinase complexes that are distinct from α4β1-mediated responses. *In vivo* inhibition of focal adhesion kinase activation partially recapitulates the $\beta1^{\Delta/\Delta}$ stress response. Mice lacking all α4 and β1 integrins (double knockouts) had, at homeostasis, the most severe phenotype with selective impairment of erythroid responses. The fact that integrins participate in mitigating stress in erythroid cells through redox activation of distinct signaling pathways by specific integrin heterodimers is a link that has not been appreciated until now.

Introduction

Reactive oxygen species (ROS) in cells play crucial roles in gene transcription, proliferation/survival of cells, and cellular defenses.[1] However, an uncontrolled production of ROS leads to many detrimental effects and cell toxicity.[2] In order to defend against ROS deleterious effects the cells are equipped with effective anti-oxidant responses in the form of ROS scavenging enzymes or other anti-oxidant molecules. Red blood cells (RBCs), compared with many other cell types, are highly susceptible to ROS generation as hemoglobin (Hb) is a significant source of superoxide generation.[3,4] At the same time, RBCs are equipped with a large supply of anti-oxidant enzymes (superoxide dismutase, catalase, peroxiredoxin, etc.) to combat increased ROS formation which, if left unbalanced, can affect plasma membrane proteins, lipid peroxidation and compromise RBC viability resulting in intra- and extra-vascular lysis.[3,4] Therefore, it is not surprising that mice with reduced cellular anti-oxidant responses[5-14] have a poorly compensated anemia and decreased erythroid responses to stress. In addition to genetic impairments in direct anti-oxidant responses (i.e. enzymes and factors involved in anti-oxidant signaling), there is a host of other genetically impaired pathways that compromise terminal erythroid differentiation or influence erythropoietin (Epo)-responses, especially after stress.[15-22] In the great majority of these cases, attempts to compensate for RBC losses lead to an increase in erythropoiesis at early stages (ineffective erythropoiesis) and to splenomegaly in mice.

Although factors influencing basal erythropoiesis, i.e. Epo, stem cell factor, GATA-1, are also required for stress erythropoiesis, it is generally believed that the latter is controlled by additional and distinct molecular networks. This view is supported by the fact that many mouse models with impaired responses in stress-related molecules (*Online Supplementary Table S1*)[5-29] have a normal basal erythropoiesis. During stress, bone morphogenetic protein 4/hedgehog signaling and hypoxia are necessary for expanding a specific subset of progenitors within the murine splenic environment to quickly address stress demands for RBC production.[17,18,20] Since the ability of these special progenitor cells to respond to stress depends on cues from the microenvironment (ME) of the spleen rather than the bone marrow (BM), distinct erythroid cell/ME interactions are apparently at play in the former but not the latter environment under stress. Although the spleen has been recognized as the favored ME for stress response in the mouse, the specific ME/hematopoietic cell interactions responsible for the differences between BM and spleen environment have not been defined. Several cellular (i.e. macrophages or endothelial cells) or extracellular matrix molecules (i.e. fibronectin, laminins, tenascin-C, glycosaminoglycans, heparin sulphate proteoglycans, etc.) are presumed to be responsible for these differences; however, no definitive conclusions have yet been made.

We have previously described a compromised response to erythroid stress in mice with β1-integrin deficiencies.[29] These mice do not mount a splenic erythroid response to hemolytic stress, but the mechanistic underpinnings of their poor stress

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response were not defined. In the present study, we evaluated a large number of mice during steady state erythropoiesis and unveiled that mice with β1 integrin deficiency have an uncompensated anemia with ineffective erythropoiesis and poor RBC survival, largely because of their susceptibility for ROS accumulation and inability to mount effective anti-oxidant responses. Beyond these effects at terminal erythroid maturation, post-stress impairment in erythroid proliferative expansion in spleen is dependent on α5β1 rather than α4β1 signaling responses. Collectively, our data shed new light on the roles of integrins in erythropoiesis both at homeostasis and after stress.

Methods

Mice and treatments

Tie2Cre+ α4f/f mice and MxCre+ β1f/f mice have been described previously.[29] MxCre+;α4f/f,β1f/f mice (Dko) were obtained by breeding α4f/f mice to MxCre+ β1f/f mice. PolyI-C treatment of these mice was carried out as described[29] for interferon-induced Cre activation. Phenylhydrazine (PHZ) treatment was carried out as described.[29] For 5-fluorouracil (5-FU) experiments, mice received a single injection of 5-FU (200 mg/kg) and recovery was followed up to 32 days. For FAK14 inhibitor experiments mice received 25 mg/Kg of FAK14 daily for four days. All experiments with mice were approved by the University of Washington Institutional Animal Care and Use Committee. Peripheral blood profiles (cell counts and erythrocyte parameters) were made using HEMAVET 950FS (DREW Scientific Inc.).

RBC life span

Mice were injected with 100 mg/kg EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) and percentages of circulating biotinylated RBCs were analyzed by FACS after staining with streptavidin-Cy5.

H_2O_2-induced RBC lysis

RBCs at 4% hematocrit were washed in phosphate buffered saline/20 mM glucose, incubated with various concentrations of H_2O_2 for 3 h at 37°C and percentages of non-lysed RBCs were determined by FACS in the live RBC gate.

Flow cytometry

Antibodies to mouse β1 integrin, α5 integrin, TER119, CD71, CD117 (c- Kit), LPAM (α4β7), phospho-p38 MAPK, phosphor-STAT5 were from BD Biosciences, anti-mouse α4, PS/2 was from Southern Biotech. Reticulocytes were measured by flow cytometry after staining an aliquot of whole blood with thiazole orange (0.1 mg/L, Molecular Probes) and gating on red blood cells.

ROS measurement

For total ROS, cells were labeled with CD71, TER119 and c-Kit antibodies, washed, loaded with CM-H2DCFDA (Molecular Probes) for 20 min at 37°C and analyzed by FACS. In some experiments, after loading with DCFDA, cells were treated with H_2O_2 (10 μM) for 20 min before FACS analysis. For mitochondrial ROS, after surface labeling, cells were washed, loaded with MitoSox Red and or MitoTracker Green (Molecular Probes) for 30 min at 37°C and analyzed by FACS.

CFU-C assays

To quantify committed progenitors of all lineages, 50,000 BM cells, 500,000 splenic cells or 50-250 microliters of peripheral blood were cultured in semi-solid methylcellulose medium supplemented with cytokines (ReachBio) for seven days and colonies were scored.

Real-time PCR

Kit-positive and TER119-positive cells were enriched by magnetic beads and RNA was isolated, reverse transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using SyberGreen reagent (Applied Biosystems) with primer pairs listed in Table 1.

Results

$\beta1^{\Delta/\Delta}$ mice have a modest uncompensated anemia and decreased RBC survival

Previous observations have shown that, for erythroid cells, the most relevant β1 integrin heterodimers are the α4β1 and α5β1.[29,30] Although our present focus is on erythropoiesis, it is important to emphasize that, in our mice, integrins were deleted in all hematopoietic cells. As indicated in Figure 1A, the expression of α4, α5, and β1 integrins was distinct in all bone marrow (BM) populations of the three mutant mice; $\alpha4^{-/-}$ mice lack α4 integrins (α4β1, α4β7) in all populations tested, whereas $\beta1^{\Delta/\Delta}$ mice lack in their erythroid cells mainly α4β1 integrins and double knockouts (Dkos) lack both α4 and α5 integrins. Furthermore, other details in expression of these integrins are observed: α5 (and β1) expression in control and $\alpha4^{-/-}$ erythroid cells declines significantly with maturation (compare $Kit^{+}CD71^{hi}$ to $CD71^{lo}/TER119^{+}$ [R3]), in contrast

Table 1. Primers for qPCR.

	Forward	Reverse
Catalase	5'-CCTCGTTCAGGATGTGGTTT-3'	5'-TCTGGTGATATCGTGGGTGA-5'
Mn SOD	5'-TTAACGCGCAGATCATGCA-3'	5'-GGTGGCGTTGAGATTGTTCA-3'
p38 MAPK	5'TGAACTTCGCAAATGTATTTATTGGT-3'	5'-ATCTGAGTCCAAAACGAGCATCT-3'
STAT5a	5'-CAGATCAAGCAAGTGGTCCC-3'	5'-CACAGGACTAGAACACCTGC-3'
FoxO3	5'-AGTGGATGGTGCGCTGTGT-3'	5'-CTGTGCAGGGACAGGTTGT-3'
NRF2	5'-CTCGCTGGAAAAAGAAGTG-3'	5'-CCGTCCAGGAGTTCAGAGG-3'
PRDX2	5'-GCACTGCCTGGTGTGGTTT-3'	5'-GCCATGACTGCGTGAGCAA-3'
HPRT	5'-GCTGGTGAAAAGGACCTCT-3'	5'-CACAGGACTAGAACACCTGC-3'
bActin	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGTAACAATGCCATGT-3'

to α4 expression. Dko mice lack all α4, α5, and β1 integrins in early and later erythroid cells. To test how the expression of these integrins affects base-line erythropoiesis in adult animals, we assessed complete blood counts (Hb, Hct, white blood cells [WBCs], platelets, reticulocytes, red cell indices and morphology) in a significant number of all the above mice. As seen in Figure 1B, both $\beta1^{\Delta/\Delta}$ and Dko mice had a modest anemia with decreased Hct and Hb, significantly different from controls, whereas the $\alpha4^{-/-}$ mice showed very modest differences from control mice. In addition, $\beta1^{\Delta/\Delta}$ and Dko mice had an increase in RBC distribution width (RDW) reflecting anisocytosis (*Online Supplementary Table S2* and *Online Supplementary Figure S1A*), and an increase in reticulocyte count (Figure 1B). These data indicate that $\beta1^{\Delta/\Delta}$ and Dkos had a partially compensated anemia, in contrast to increased numbers in other lineages (WBCs and platelets) (*Online Supplementary Figure S1B*). To test whether we are dealing with enhanced destruction rather than inadequate production of RBCs, we evaluated RBC survival and production parameters in BM and spleen, i.e. cellularity, progenitor level and nucleated erythroid cell representation in BM and spleen. The survival of RBCs in $\beta1^{\Delta/\Delta}$ and Dkos was reduced by over 50% compared to controls, whereas $\alpha4^{-/-}$ mice had better survival (Figure 1B). Furthermore, the relative proportion of early erythroid cells (R1 and R2) was higher in $\beta1^{\Delta/\Delta}$ and Dko mice compared to later cells (R3 and R4) in contrast to controls and $\alpha4^{-/-}$ mice (Figure 1C). These data indicate that the maturation profile of erythroid cells was altered in $\beta1^{\Delta/\Delta}$ and Dkos, as greatly diminished proportions of late or mature erythroblasts were present. It is of note that the total cellularity, total $Kit^+/CD71^+$ and total progenitor content in both BM and spleen were not impaired, being slightly higher than controls (*Online Supplementary Figure S1B*) Collectively, the data focusing on erythroid cells suggest that impairment in accumulation of late maturation forms of erythroid cells is responsible for the uncompensated anemia in $\beta1^{\Delta/\Delta}$ and Dko, rather than insufficient production from earlier progenitors.

Erythroid cells from $\beta1^{\Delta/\Delta}$ and Dko mice have increased ROS

As the RBC survival was significantly shortened in $\beta1^{\Delta/\Delta}$ and Dko mice, we tested whether the short survival was

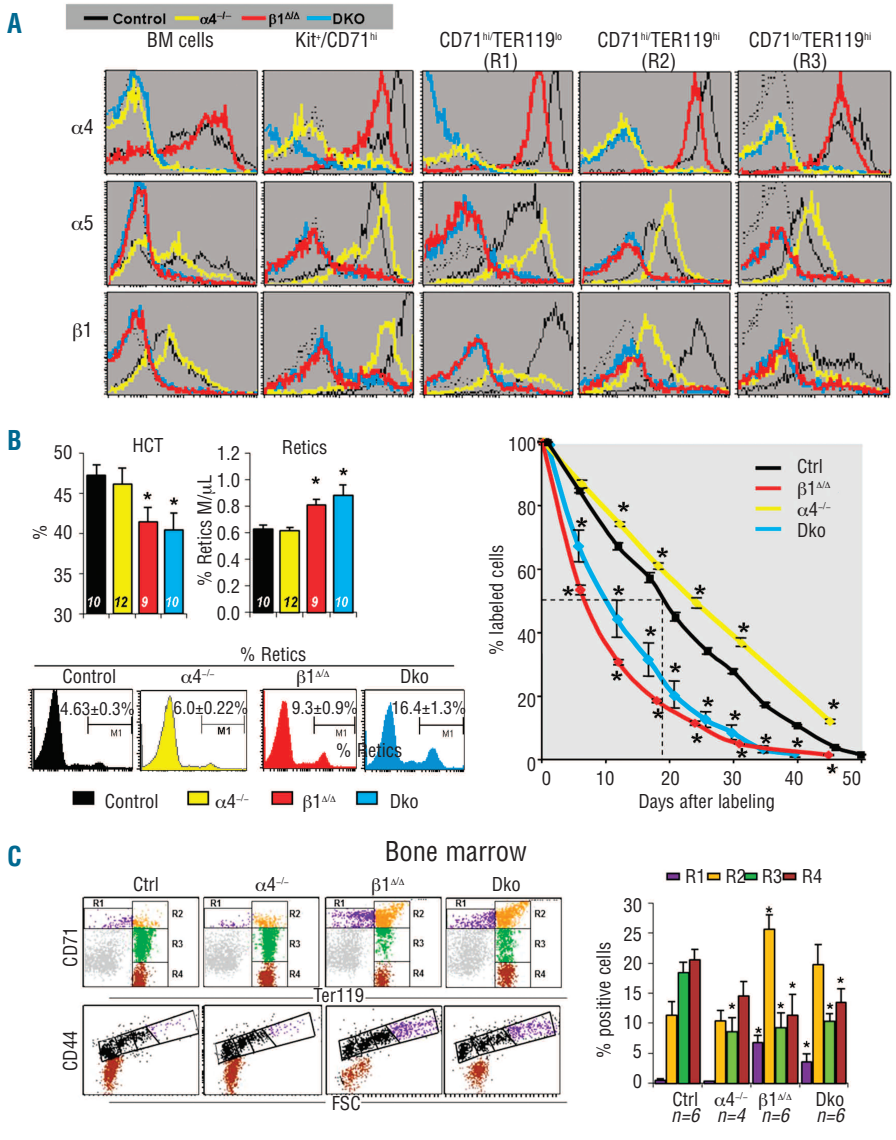

Figure 1. Hematopoiesis at homeostasis in integrin-deficient mice. (**A**) Expression of ββ1, α4 andαα5 integrins in unfractionated BM cells (left three vertical panels), in c-$Kit^+/CD71^{hi}$ cells and in erythroid cells of increasing maturity R1 to R3 (*see below*). Black solid line: control; yellow line: $\alpha4^{-/-}$; red line: $\beta1^{\Delta/\Delta}$; turquoise line: Dko; black dotted line: isotype control. (**B**) Hematocrit levels, reticulocyte numbers per mL PB and reticulocyte percentage in peripheral blood (left panel, numbers within bars indicate number of mice used in a corresponding experiment) and RBC life span (right panel) at steady state in control (n=3), $\alpha4^{-/-}$ (n=5), $\beta1^{\Delta/\Delta}$ (n=5) and Dko (n=4). (**C**) Erythroid maturation in bone marrow at steady state. Left panel: Fluorescence Activated Cell Sorting (FACS) profile of BM cells using Ter119 and CD71 antibody-based strategy (upper left), and CD44 antibody-based strategy (bottom left). In dot blots where CD44/FSC profile is shown, proerythroblasts (R1 cells) are in purple, and RBC are in brown; all the other stages of erythroid maturation are in black). Right panel: percent of cells at various stages of maturation (R1=$CD71^{hi}$,$Ter119^{lo}$, purple; R2=$CD71^{hi}$, $Ter119^+$, orange; R3=$CD71^{lo}$, $Ter119^+$, green, R4=$CD71^-$,$Ter119^+$, RBC, brown). *Indicates a significant difference over control, $P<0.05$, calculated with Excel in Figures 1-3.

due to ROS accumulation in both reticulocytes ($CD71^+/TER119^+$) and mature RBCs ($CD71^-/TER119^+$) in PB. At homeostasis, there was a trend for higher ROS levels in $\beta1^{\Delta/\Delta}$, but significant differences were seen only in Dko mature RBCs compared to controls or $\alpha4^{-/-}$ RBCs. However, after incubation with low doses of peroxide *in vitro*, a significant ROS increase was seen in both $\beta1^{\Delta/\Delta}$ and Dkos, but not in $\alpha4^{-/-}$ cells (Figure 2A). The high susceptibility of Dko RBCs to oxidative stress was also consequential in H_2O_2-induced RBC lysis experiments, where increased lysis of Dko RBCs was detected at all H_2O_2 doses used, whereas RBCs from $\alpha4^{-/-}$ and β1-deficient mice showed increased lysis only at high doses of H_2O_2 (Figure 2A). These data suggest that the red cells of Dko mice are very sensitive to oxidative stress, much more than $\beta1^{\Delta/\Delta}$ and $\alpha4^{-/-}$ cells, likely because of additional membrane changes (*Online Supplementary Figure S1A*).

Mature RBC cells more than any other cells are vulnerable to oxidation or ROS-induced damage because of Hb accumulation. To inquire whether even earlier cells in erythroid development were showing abnormal ROS levels, we assessed ROS levels in $TER119^+$ erythroblasts and kit^+ cells in BM. ROS levels in $TER119^+$ cells were significantly increased in all mutant mice compared to controls (Figure

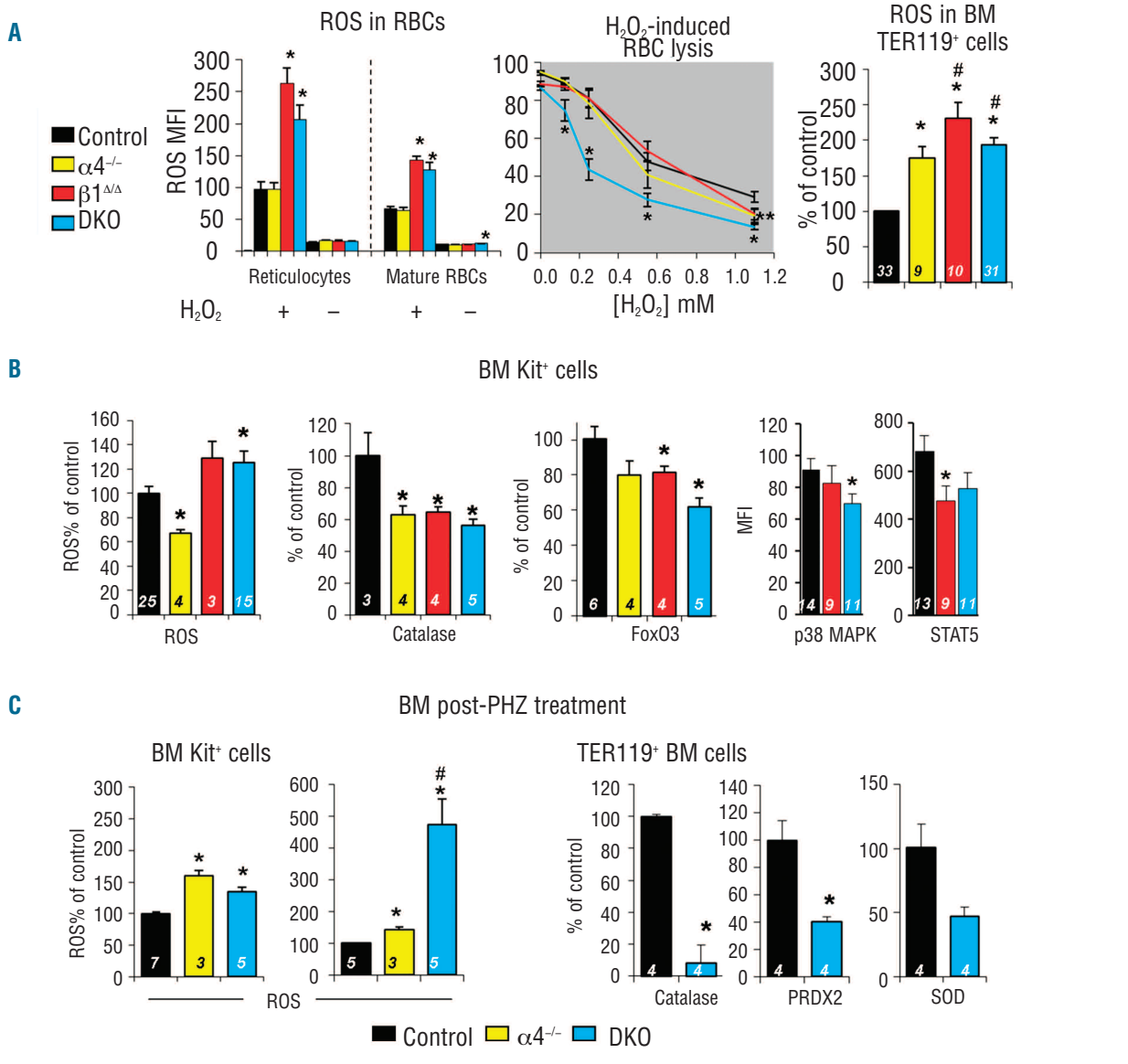

Figure 2. ROS studies at homeostasis and after challenge in integrin-deficient mice. (A) ROS levels in RBC ($Ter119^+$, $CD71^-$) and reticulocytes ($Ter119^+$, $CD71^+$) in peripheral blood at steady state and after challenge with H_2O_2 (10 μM, 20 min) *in vitro*, as measured by DCFDA oxidation by FACS (left panel); resistance of RBC to H_2O_2-induced lysis as measured by percentage of live RBCs analyzed by FS/SSC FACS profile after incubation with increasing H_2O_2 concentration (middle panel); ROS levels in BM $Ter119^+$ (right panel). (B) $c\text{-}Kit^+$ BM cells at steady state: Total ROS levels (left panel); relative expression of Catalase and FoxO3 as measured by qPCR (middle panel); levels of activated p38 MAPK and STAT5 as measured by FACS using anti-phospho p38 MAPK and anti-phospho STAT5 mabs (BD Biosciences). (C) Response to PHZ-induced stress: ROS levels in BM Kit^+ (left panel) and $TER119^+$ (middle panel) cells and expression of anti-oxidant enzymes in $TER119^+$ BM cells (right panel). Throughout the figure, Control mice (black lines or bars), α4 mice (yellow lines or bars), β1 mice (red lines or bars), and Dko mice (turquoise lines or bars) are shown. Numbers in bars indicate the number of mice used in a corresponding experiment. *Indicates significant difference over control, and #indicates significant difference over $\alpha4^{-/-}$, $P<0.05$.

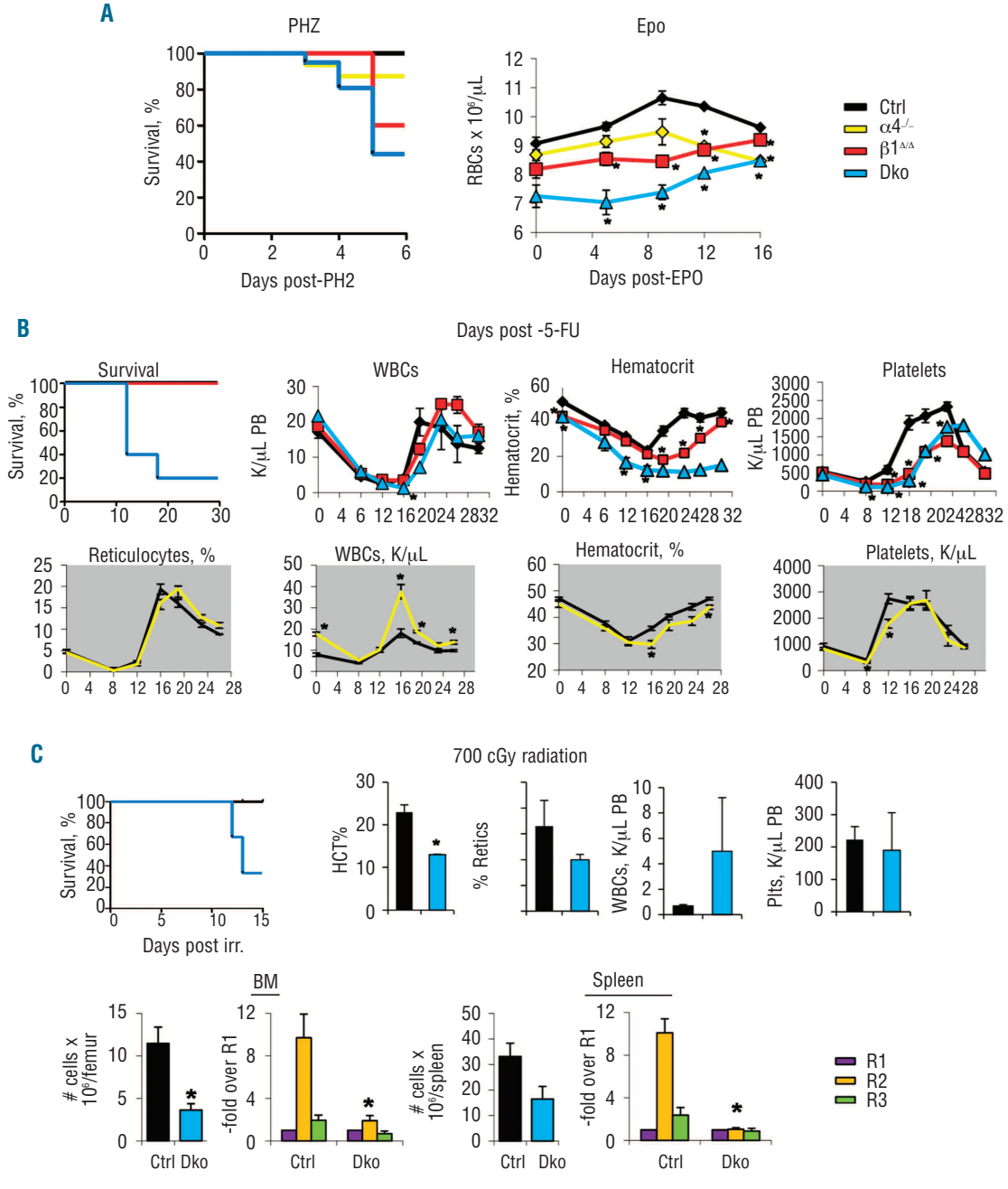

Figure 3. Stress treatments (5-FU, irradiation, Epo and PHZ) in Dko and comparisons with other mutant models. (A) Left panel: response to PHZ-induced acute anemia. Control and integrin-deficient mice received PHZ injections (60 mg/kg, i.p. on two consecutive days). Survival of mice after PHZ challenge is shown. Ctrl, n=20; $\alpha4^{-/-}$, n=16; $\beta1^{\Delta/\Delta}$, n=15; Dko, n=17). Survival statistics for all mutant mice in A, B and C were generated using the log rank (Mantel-Cox) Test, and were found to be significant over control, $P<0.05$. Right panel: response to Epo treatment. Control and integrin-deficient mice received injections of human recombinant erythropoietin (50 U, i.p. on days 1, 3 and 5) and were followed for 16 days. Samples of PB were drawn and total RBC numbers and hematocrit levels were determined. Control mice n=5; $\alpha4^{-/-}$ n=6; $\beta1^{\Delta/\Delta}$ n=4; Dko n=3. (B) Response to a single injection of 5-FU. Upper panel: Lethally irradiated wild type mice were reconstituted with control, $\beta1^{\Delta/\Delta}$, or Dko bone marrow cells and left to recover for 12 weeks. Reconstituted mice (5 mice per group) received a single injection of 5-FU (200 mg/kg i.p.) and were followed for a month after injection. $P<0.05$ for survival differences. PB was drawn before injection, on day 8 and every 3-4 days after that. WBC, hematocrit levels and platelet counts are shown. Lower panel: $\alpha4^{-/-}$ (n=6) and corresponding control mice (n=3, black line) were treated with 5-FU and followed for a month after injection exactly as the transplanted mice. $\alpha4^{-/-}$ mice (lower panels) in contrast to Dkos, were able to recover their hematocrit levels, albeit with delayed kinetics. (*$P<0.05$) (C) Response to sub-lethal irradiation. Upper panel: primary ablated Dko mice (n=6) and controls (n=6) received 700 cGy whole body irradiation and were sacrificed 15 days later. By the time of sacrifice only two Dko mice survived the challenge, whereas in the control group all animals survived. Peripheral blood samples were analyzed by Hemavet: the levels of circulating WBCs and Platelets as well as Hematocrit levels are shown. Percentage of Reticulocytes in PB was determined by FACS following staining with thiazole orange. Lower panel: BM and spleen cellularity as well as erythroid maturation profiles in BM and Spleen are also shown. Throughout the Figure, controls are shown in black, $\alpha4^{-/-}$– in yellow, $\beta1^{\Delta/\Delta}$ in red and Dko in turquoise. *Indicates significant difference over control, $P<0.05$.

2A), whereas ROS in Kit$^+$ cells were higher in $\beta1^{\Delta/\Delta}$ and Dko mice (Figure 2B). Furthermore, mitochondrial-derived ROS was increased in $\beta1^{\Delta/\Delta}$ and Dkos suggesting that, at least in part, increasing ROS production seen in these cells was of mitochondrial origin (*data not shown*).

Taken together, our data on RBCs and TER119$^+$ erythroblasts show detectable increases in ROS levels at homeostasis, especially in $\beta1^{\Delta/\Delta}$ and Dko mice with $\alpha4^{-/-}$ showing the mildest effects. Excessive levels of ROS can be due to either hyperproduction of ROS, or to its accumulation largely because of inadequate anti-oxidant defenses.

Anti-oxidant enzymes and signaling molecules associated with redox responses are impaired in integrin deficient mice

ROS scavenging enzymes (i.e. catalase, peroxiredoxin [PRDX2], superoxide dismutase [SOD], etc.), whether mitochondria-derived or not, are synthesized at earlier stages of differentiation and carried through to mature enucleated cells. Given this, we assessed levels of these enzymes in Kit$^+$ cells containing earlier erythroid progenitor cells. As shown in Figure 2B, catalase mRNA levels were reduced in Kit$^+$ cells at steady state in all mice. Furthermore, FoxO3,[6] a transcription factor responsible for the activation of anti-oxidant enzymes including catalase was also reduced (not significantly in $\alpha4^{-/-}$ mice) compared to controls (Figure 2B and *Online Supplementary Figure S2A and B*). Two other molecules relevant to ROS responses, p38 MAPK[31] and Stat5[6,16] were modestly decreased or unchanged in $\beta1^{\Delta/\Delta}$ and Dko mice in contrast to expected increases as redox sensors (Figure 2B). Nrf$_2$[12] was also no different from controls (*data not shown*). Moreover, it is important to emphasize that after oxidative stress induced by PHZ treatment, the differences from controls in catalase and other anti-oxidant enzymes (PRDX2 and SOD) were exaggerated, especially in Dkos, in which even lower levels were present after stress (Figure 2C). These data emphasize the inability of integrin-deficient erythroid cells to cope with any increase in ROS levels because of inappropriate responses in several signaling molecules participating in anti-oxidant defenses. Most importantly, when mRNA arrays were performed using Kit$^+$ cells at homeostasis from controls, $\alpha4^{-/-}$ or $\beta1^{\Delta/\Delta}$ mice (*Online Supplementary Table S3*), the levels of several ROS-scavenging enzymes were decreased in $\beta1^{\Delta/\Delta}$ providing an independent confirmation of these findings.

α4 versus α5 integrins likely elicit different signaling responses post stress

One of the distinct differences between $\alpha4^{-/-}$ compared to $\beta1^{\Delta/\Delta}$ and Dko mice is the splenic response to erythropoietic stress.[29] Stress-driven erythroid expansion is not impaired in the spleen of $\alpha4^{-/-}$ mice.[29] This would indicate that proliferative expansion of early erythroid cells is independent of α4-expression, but their proliferation and or survival requires α5β1 integrins. Do these two α-integrins elicit different signaling pathways responsible for stress-induced erythroid proliferation in spleen?

Focal adhesion kinase (FAK) is a nodal integrator of signal transduction from integrin-enriched focal adhesion sites mainly responsible for propagation of integrin-dependent signals.[32] However, signaling pathways activated by specific integrins are distinct and not necessarily FAK-dependent.[33] For example, it has previously been shown that α5β1 and α4β1 integrins stimulate motility in fibroblasts, neural cells or macrophages through distinct mechanisms: an FAK/Pyk2 linkage is essential for α5-signaling, but not for α4-dependent signaling, which depends only on c-Src activation,[34,35] and paxillin can promote α4-mediated macrophage chemotaxis independently of FAK.[36] Consequently, it could be suggested that, for erythroid proliferative expansion in spleen, as occurs in $\alpha4^{-/-}$ mice, the α4/c-Src-dependent signaling is dispensable. If instead the α5/FAK-dependent signaling is responsible, then inhibition of this signaling should phenocopy the response of $\beta1^{\Delta/\Delta}$ mice to stress. To test this possibility, we treated normal mice with the FAK inhibitor FAK14 and tested their response to PHZ-induced stress. The response in the treated mice was significantly impaired as judged by decreased erythroid expansion in spleen (with much less effect on erythroid maturation) (*Online Supplementary Figure S3A*). These data would support our thesis that the splenic proliferative response is dependent on α5β1/FAK-mediated signaling. As there is a feedback loop between FAK and p53 regulation, we also tested p53 activity in Dko erythroid cells (TER 119$^+$) post-PHZ stress (*Online Supplementary Figure S2C*). Increased cytoplasmic p53 levels were seen in Dko cells compared to controls. This result is consistent with the view expressed previously[37] that FAK binds and sequesters p53 from apoptotic signaling.[38] This finding also introduces p53 dysregulation and crosstalk with α5β1 function, as described for glioma cells,[39] however further studies are needed to consolidate these findings.

Dko mice have a more severe phenotype than $\beta1^{\Delta/\Delta}$ mice

Although there was a significant phenotypic variability among Dko animals, the average phenotype of Dko mice at homeostasis appeared somewhat more severely compromised than that of $\beta1^{\Delta/\Delta}$ mice. Differences between $\beta1^{\Delta/\Delta}$ and Dkos have important implications in data interpretation. As seen in Figure 1A, $\beta1^{\Delta/\Delta}$ mice display significant levels of α4-integrin on their surface, almost half or more of which is in the form of α4β7 (antibodies for anti-α4 always show higher levels than Abs against α4β7, but α4β7 is virtually absent in erythroid cells). For example, if Dkos are not very different or marginally different from $\beta1^{\Delta/\Delta}$ mice, then the contribution of any α4-integrin present in $\beta1^{\Delta/\Delta}$ cells to the phenotype of Dkos should also be marginal at best. If, however, Dkos are significantly different from $\beta1^{\Delta/\Delta}$ mice, then the phenotype of Dkos uncovers an important combinatorial contribution of α4-integrin in the phenotype of these mice.

To provide further evidence or to seek a clear-cut difference among $\beta1^{\Delta/\Delta}$ and Dko mice, we generated further data, either after acute stress (a single PHZ injection or single high Epo injection), or after 5-FU or irradiation induced injury to proliferating cells.

Treatment of Dkos with PHZ or with Epo were suggestive for more severe impairment in Dko mice, since these mice started to die at earlier times or responded somewhat less to Epo than did $\beta1^{\Delta/\Delta}$ mice (Figure 3A). However, the data after a single 5-FU treatment or after 700 cGy irradiation were more informative. Both approaches are dependent on regeneration from earlier progenitor cells than the acute hemolytic stress, i.e. post-PHZ, which leaves the late progenitors and Epo-dependent erythropoiesis intact. There was a significant difference in sur-

vival after 5-FU treatment since less than 20% of the Dko animals survived the treatment in contrast to 100% survival in $\beta1^{\Delta/\Delta}$ mice (Figure 3B). More revealing was the fact that the Dko animals died because they were unable to raise their Hct levels. In contrast to the RBCs, white cell and platelet recovery was no different in $\beta1^{\Delta/\Delta}$ *versus* Dko. Similar responses were revealed after 700 cGy (Figure 3C). There was a selective reduction in erythroid responses (Hct, reticulocytes, erythroid cells) in BM and spleen, but WBCs and platelets recovered promptly in Dko mice (Figure 4C). Taken together, the data clearly indicate that erythroid responses are severely compromised in Dko mice. As post-stress erythroid progenitor expansion was largely unaffected in BM and spleen in $\alpha4^{-/-}$ mice (in contrast to $\beta1^{\Delta/\Delta}$ and Dkos),[29] it follows that post stress, any combinatorial effect of α4 is exercised either at the progenitor level, pointing to a newly uncovered influence not seen in $\alpha4^{-/-}$ because of α5 redundancy, or/and at the terminal maturation level in which a defect, albeit of different severity, is seen in both $\alpha4^{-/-}$ and $\beta1^{\Delta/\Delta}$ mice. The data post 5-FU and irradiation favor the latter possibility because of selective severity in erythroid response. It is worth emphasizing that in both the $\beta1^{\Delta/\Delta}$ and Dko mice the effects seen were by and large cell autonomous, since in 5-FU experiments normal recipients reconstituted with Dko or $\beta1^{\Delta/\Delta}$ cells were used (Figure 3B).

Discussion

α5 and α4 integrins in erythropoiesis: early versus late effects

Several pieces of evidence presented here document that $\beta1^{\Delta/\Delta}$ or Dko mice have an uncompensated anemia at homeostasis with signs of ineffective erythropoiesis and shortened RBC survival likely because of their inability to counteract chronic ROS accumulation. As a result, membrane changes through protein oxidation and lipid peroxidation would affect membrane fluidity and stability,[3,4] leading to hemolysis. Since a similar picture is not seen in the absence of only α4-integrins ($[\alpha4\beta1;\alpha4\beta7]^{-/-}$) the data would suggest that the absence of other integrin heterodimers in $\beta1^{\Delta/\Delta}$ or Dkos alone or in combination are responsible for this phenotype.

Integrins expressed in differentiated erythroid cells (mainly α4β1 and α5β1) and their interactions with fibronectin (Fn) in their ME have been previously emphasized as critical for completing terminal maturation steps.[30,40,41] Specifically, on the basis of *in vitro* studies using fetal liver cells, it was concluded that Epo and Fn regulate distinct steps in the erythroid differentiation process; an early, Epo-dependent /integrin independent step, and a late Epo-independent/integrin-dependent one. Furthermore, by using Fn fragments engaging either α4- or α5-integrins or both, it was concluded that only α4, not α5, supports terminal differentiation. Thus only α4-integrin was thought to be responsible for protecting erythroid cells from apoptosis (through BcL-xL) during the Epo-independent phase of erythropoiesis.[41] In contrast to these data, Kapur *et al.*,[40] using Kit ligand-mediated signaling during G1E-ER2 GATA-1 cell differentiation, concluded that α5 and α4 have opposing effects on the growth and survival of erythroid cells as they differentiate; there was greater proliferation in cultures containing α5β1 adhesion sites compared to cells grown in suspension, or in cultures containing α4β1 adhesion sites. These results were paralleled by activation of FAK by α5β1 and its reduction by engaging α4β1. Several of our *in vivo* findings with genetically deficient mice are at variance with some of the prior conclusions. Our data emphasize the importance of α5β1 in proliferative expansion of erythroid cells post stress and, in that respect, are consistent with those of Kapur *et al.*[40] However, our data do not confirm the opposing proliferative effects of α4 *versus* α5 described in the latter study.[40] Further, our data are consistent with an influence of α4-integrin on terminal erythroid maturation, as stated by Hattangadi *et al.*,[41] but they disaffirm their conclusions about any role for α5-integrin. There are several caveats concerning these prior studies that may explain the differences. Hattangadi *et al.*[41] based their conclusions on in vitro studies using fetal liver cells cultured in the presence of Epo or Fn and relied exclusively on the use of function-blocking Abs. It is possible that the expression of α5β1 is different (possibly lower)[42] in late stages of fetal erythroblasts compared to adult BM cells, or that the expression of α5, in contrast to α4, is lost earlier in *in vitro* cultured erythroid cells compared to their non-cultured *in vivo* counterparts. Kapur *et al.*[40] used G1E immortalized (not primary) cells and specific recombinant fibronectin fragments. Nevertheless, it is important to emphasize that in our *in vivo* studies, it is unclear at what stage of erythroid differentiation the α5-dependent proliferative signaling is exerted, i.e. on c-Kit$^+$ or later Epo-dependent stages. In our studies, β1-integrins are deleted at the level of stem/progenitor cells, so that the α5-dependent events, whether exercised early or late, cannot be discerned. Definitive answers can only be obtained when the relevant integrins, alone or in combination, are deleted only in erythroid cells and not in earlier progenitors. Furthermore, the role of ME, especially altered macrophage function, on the severity of base-line phenotype in $\beta1^{\Delta/\Delta}$ or Dko compared to $\alpha4^{-/-}$ mice, will require further reciprocal transplantation experiments to settle the issue.

Integrin-dependent signaling in erythroid cells

How are the phenotypic differences between α4- and α5-integrins molecularly mediated? Is the redox-triggered α5β1 *versus* α4β1 signaling in erythroid cells distinct? Very little is known about the precise downstream signaling pathways that are integrin-dependent in erythroid cells, although more is known in non-hematopoietic cells.[33,35,43-48] Thus, oxidative stress is assumed to modulate cell-to-cell adhesion by affecting expression of adhesion molecules (i.e. α5 to Fn or αv to vitronectin).[43-47] Furthermore, in fibroblasts, neural cells or macrophages, migratory signals elicited by α4β1 are distinct from those elicited in the same cells by α5β1.[33-36] Their motility was dependent on α5/FAK signaling, but this was dispensable for α4β1-mediated signaling mediated through c-Src activation instead. In hematopoietic cells, integrin-dependent FAK signaling is thought to be important in integrating growth/survival signals, especially under stress.[28] FAK is activated by integrins and is responsible for amplifying integrin induced signals. $FAK^{-/-}$ mice show impairment in both Kit- and Epo-directed signals, although there are controversial data on this issue.[49] Since in our mice proliferative responses in the spleen appear to be dependent on α5β1 and not α4β1, it follows that after engagement of α5β1 integrins, the FAK-recruited complexes and their downstream initiated cascades are likely involved (*Online Supplementary Figure S3B*).

The fact that treatment of normal mice with an anti-FAK inhibitor partially phenocopies the response of $\beta1^{\Delta/\Delta}$ mice post stress supports this view (*Online Supplementary Figure S3A*). It is also intriguing that mice with deletion of Src kinase Lyn, SHP1 and SHIP-1 phosphatases[50] have a similar phenotype to that of $\alpha4^{-/-}$ mice, suggesting that progenitor expansion is not dependent on Src signaling. Future studies are needed to obtain further molecular details that involve redox-based activation of α5/FAK signaling and their downstream effectors, particularly in genetic models with integrin ablation only in erythroid cells.

Phenotype of Dko mice

In addition to differences between α4- and β1-deficient mice, results in mice with complete absence of all α4- and β1-dependent integrins (Dko), presented for the first time in the present study, provide an added insight on how integrins affect erythropoiesis. The Dko mice have a more severe phenotype than the $\beta1^{\Delta/\Delta}$ mice both at homeostasis (Figure 1) and after stress (Figure 3), indicating that the existing α4 on the surface of $\beta1^{\Delta/\Delta}$ cells, as seen with anti-α4 Abs (Figure 1A), may contribute to some amelioration of the phenotype. But this is exacerbated in the complete absence of both α5 and α4 integrins in all cells, including all stages of erythroid cells. Interestingly, in Dko mice the impairment in oxidative status is readily demonstrable not only in RBCs, but in earlier cells (Kit^+), leaving the terminally mature RBCs ill-equipped to address oxidative stress. Since deletion of only α4 integrins mainly affects terminal maturation events, these are likely exacerbated because of complete absence of α4 and α5 integrins in Dko mice both at homeostasis and after stress. It is also important to emphasize that the overall survival of both $\beta1^{\Delta/\Delta}$ and Dko mice is severely compromised compared to $\alpha4^{-/-}$ mice likely because of β1 ablation in non-hematopoietic cells (microenvironmental stromal cells, endothelial cells) and health deterioration. To address the full contribution of integrin-deficient ME effects on erythropoiesis in addition to intrinsic effects of β1-integrins, detailed studies in reciprocal transplantation experiments are needed. Moreover, studies in mice with deletion of integrins only in erythroid cells, in contrast to currently studied mice, will ultimately delineate the functional behavior of integrin-deficient erythroid cells.

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