TLR7 ligation augments hematopoiesis in Rps14 (uS11) deficiency via paradoxical suppression of inflammatory signalling.

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Abstract:
Myelodysplastic syndrome (MDS) is a haematological malignancy characterised by blood cytopenias and predisposition to acute myeloid leukaemia (AML). Therapies for MDS are lacking, particularly those that impact the early stages of disease. We developed a model of MDS using zebrafish using knockout of Rps14, the primary mediator of the anaemia associated with del (5q) MDS. These mutant animals display dose- and age-dependent abnormalities in haematopoiesis, culminating in bone marrow failure with dysplastic features. We utilized rps14 knockdown to undertake an in vivo small molecule screen to identify compounds that ameliorate the MDS phenotype, identifying imiquimod, an agonist of TLR7 and TLR8. Imiquimod alleviates anaemia by promoting haematopoietic stem and progenitor cell expansion and erythroid differentiation, the mechanism of which is dependent on TLR7 ligation and Myd88. TLR7 activation in this setting paradoxically promoted an anti-inflammatory gene signature indicating crosstalk between pro-inflammatory pathways endogenous to Rps14 loss and NFκB pathway via TLR7.

Finally, we show that in highly purified human bone marrow samples from anaemic patients, imiquimod leads to an increase in erythroid output from myelo-erythroid progenitors and common myeloid progenitors. Our findings have both specific implications for the development of targeted therapeutics for del (5q) MDS and wider significance identifying a potential role for TLR7 ligation in modifying anaemia.

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TLR7 ligation augments haematopoiesis in Rps14 (uS11) deficiency via paradoxical suppression of inflammatory signalling and enhanced differentiation

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Running Title: TLR7 in a del (5q) MDS
Abstract

Myelodysplastic syndrome (MDS) is a haematological malignancy characterised by blood cytopenias and predisposition to acute myeloid leukaemia (AML). Therapies for MDS are lacking, particularly those that impact the early stages of disease. We developed a model of MDS using zebrafish using knockout of Rps14, the primary mediator of the anaemia associated with del (5q) MDS. These mutant animals display dose- and age-dependent abnormalities in haematopoiesis, culminating in bone marrow failure with dysplastic features. We utilized rps14 knockdown to undertake an in vivo small molecule screen to identify compounds that ameliorate the MDS phenotype, identifying imiquimod, an agonist of TLR7 and TLR8. Imiquimod alleviates anaemia by promoting haematopoietic stem and progenitor cell expansion and erythroid differentiation, the mechanism of which is dependent on TLR7 ligation and Myd88. TLR7 activation in this setting paradoxically promoted an anti-inflammatory gene signature indicating crosstalk between pro-inflammatory pathways endogenous to Rps14 loss and NFκB pathway via TLR7. Finally, we show that in highly purified human bone marrow samples from anaemic patients, imiquimod leads to an increase in erythroid output from myelo-erythroid progenitors and common myeloid progenitors. Our findings have both specific implications for the development of targeted therapeutics for del (5q) MDS and wider significance identifying a potential role for TLR7 ligation in modifying anaemia.

Key Points

Rps14-deficient zebrafish demonstrate features of MDS and impaired stress responses.

Rps14-induced anaemia is rescued on ligation of Tlr7, via paradoxical suppression of inflammatory signalling and enhanced differentiation.
Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid malignancies associated with cytopenias and evolution to acute myeloid leukaemia (AML). MDS with loss of all or part of the long arm of chromosome 5 (del(5q) MDS) is the most common subtype of MDS and, importantly, 5q loss has been shown to be an initiating event in MDS development in the majority of such cases.\(^1\),\(^2\),\(^3\)

The only curative therapy for patients with MDS remains stem cell transplantation, for which many older MDS patients are unfit. Thus, there remains an unmet need for novel therapies for del (5q) MDS.

Ribosomal protein of the small subunit, *RPS14* (also now known as uS11\(^4\)), located in the critically deleted region (CDR) of chromosome 5q, is one of several genes identified as a haploinsufficient tumor suppressor gene (TSG) in del(5q) MDS.\(^5\)-\(^10\) We and others have previously shown haploinsufficient levels of Rps14 recapitulate features of del(5q) MDS in zebrafish, mice and primary human cells.\(^5\),\(^11\),\(^12\)

Proposed mechanisms for Rps14-mediated haematopoietic defects include p53-dependent apoptosis and increased pro-inflammatory innate-immune signalling via toll-like receptor 4 (TLR4) resulting from an increase in translation of cognate ligands S100A8 and S100A9.\(^11\) Additionally, activation of TLR-MyD88-NFκB(NF\(\kappa\)B) signalling in del(5q) MDS results from haploinsufficiency of other genes and microRNAs contained within the 5q CDR.\(^7\),\(^9\) However, signaling through TLR4 is also required for the generation of haematopoietic stem and progenitor cells (HSPC) in both steady state and stressed haematopoiesis.\(^13\)-\(^15\) This highlights that homeostatic regulation of inflammatory signalling through TLR pathways is critical to maintain normal haematopoiesis.

We created a novel zebrafish model of del (5q) MDS using transcription activator-like effector nucleases (TALENs) to mutate *rps14*. We used Rps14-deficient zebrafish to identify small molecules that alleviate anaemia in this model and identified the TLR7/8 agonist imiquimod. We show that this effect is dependent on TLR7, and results in improved haemoglobinisation and an increase in HSPC. Importantly, imiquimod leads to an increase in erythroid output in both fish and primary human cells from anaemic patients, an effect not restricted to but enhanced by the haploinsufficiency of Rps14. Analysis of Rps14-deficient HSPC shows upregulation of negative regulators of canonical WNT signalling, which is reversed following imiquimod exposure. Furthermore we show
paradoxical down-regulation of inflammatory signalling and NFκB target genes in Rps14h−/− exposed to imiquimod, that is dependent on Myd88. These data suggest crosstalk between the endogenous pro-inflammatory effects of Rps14 haploinsufficiency and TLR7 activation leads to attenuation of anaemia in our model.

Methods

Zebrafish husbandry

Zebrafish (Danio rerio) AB, AB/TL and transgenic strains Tg(gata1:DsRed), Tg(itga2b:GFP), Tg(NFκB:GFP)sh235 were maintained according to standard procedures and UK Home Office guidelines16,17,18,19. Embryos were staged according to Kimmel20 and expressed in hours/days post-fertilization (hpf/dpf).

Generation of a zebrafish rps14 mutant line

TALENs targeting exon 2 of the zebrafish rps14 gene were made using FLASH21.

Morpholinos

Morpholinos(MO) targeting the 5′UTR/ATG codon (gata1) or splice donor sites (rps14, myd88) or Gene-tools standard control were injected into 1-2 cell stage embryos at doses previously described12,22,23.

Immunohistochemistry

O-dianisidine staining of haemoglobin and Sudan Black(SB) staining to visualise granulocytes were carried out as previously described24,25. SB positive cells were quantified from the distal end of the yolk extension to the tail tip in the caudal haematopoietic tissue(CHT; fetal liver equivalent in zebrafish).

Induction of stress

Haemolytic stress was induced by exposure of embryos to phenylhydrazine(PHZ)26. Embryos were incubated in 1 µg/mL PHZ from 24 hpf to 48 hpf and then washed. Cold stress was induced following gastrulation by placing 6 somite stage embryos at 22°C.

Microscopy
Microscopy was performed using a Leica M205 FA stereomicroscope using a Leica DFC310 FX camera and LAS 4.0 software. All images were processed with Fiji version 2.0.0-rc- 43/1.51f or Photoshop CS6.

Statistical analysis

Data are presented as mean values ± standard deviation (SD). Analysis was performed using GraphPad Prism (GraphPad Software, La Jolla California USA). Specific statistical tests are shown in legends.

Zebrafish by flow cytometry

Individual embryos and adult zebrafish kidneys were analysed by flow cytometry as described previously\(^{12}\).

Cytospins

\(1 \times 10^5\) cells were centrifuged onto slides, fixed with methanol and then stained with May-Grunwald-Giemsa stain. Images were taken with Hamamatsu Nanozoomer 2.0 RS

Western blotting

Protein lysates were obtained from pooling 6dpf genotyped embryos. Blots were probed with Phospho-eIF2α (Ser51)(119A11) Rabbit mAb #3597(Cell Signalling) and Total EIF2α ab26197(Abcam).

Results

Rps14 loss results in dose-dependent haematopoietic abnormalities

We have previously shown that knockdown of Rps14 using MO results in anaemia, modelling the erythroid defect of del (5q) MDS\(^ {12}\). We used TALENs to generate a stable Rps14 mutant zebrafish with an early frameshift mutation (\(rps14^{E8fs}\))(Figure S1). Using whole mount immunofluorescence, we demonstrated loss of Rps14 protein in an allelic dose-dependent manner, indicating that Rps14\(^{E8fs/-}\) animals have haploinsufficient protein levels modelling that seen in del (5q) MDS(Figure S2). Rps14\(^{E8fs/E8fs}\), herein referred to as Rps14\(^{-/-}\), show profound developmental anomalies and are lethal by 5dpf. O-dianisidine staining at 4dpf showed that Rps14\(^{-/-}\) embryos have markedly reduced haemoglobinisation(Figure 1C) compared to Rps14\(^{+/+}\)(Figure 1A). However, developmental morphology and haemoglobinisation in Rps14\(^{-/-}\) embryos was
indistinguishable from their Rps14<sup>-/-</sup> siblings (Figure 1A, B). To quantify erythroid cells, we used Rps14<sup>E8fs</sup> carrying Tg(gata1:dsRed). Importantly, flow cytometry of these embryos demonstrated a decrease in numbers of dsRed-expressing erythroid cells at 3dpf (Figure 1D, S3A). This indicates that Rps14<sup>-/-</sup> embryos have an anaemia associated with reduced red cell number yet are able to maintain adequate haemoglobinisation.

We next determined the impact of Rps14 loss on definitive myelopoiesis. Using sudan black (SB) to stain granulocytes at 4dpf, we identified a reduction in SB-positive granulocytes located within the CHT (Figure 1K) in a dose-dependent manner with allelic loss of Rps14 (Figure 1E-H).

We then assessed the effects of Rps14 loss on HSPC utilizing Tg(itga2b:GFP) in which GFP<sup>lo</sup> cells in the CHT label HSPC<sup>18</sup>. HSPC quantification in the CHT at 4dpf of Rps14<sup>-/-</sup> compared to Rps14<sup>+/+</sup> did not demonstrate any significant difference (Figure 1I-L, S3B). Rps14<sup>-/-</sup> have virtually absent HSPC and extensive autofluorescence and were not assessable. We further assessed HSPC numbers using whole mount in situ hybridisation (WISH) of c-myb in the CHT at 4dpf. By contrast to Tg(itga2b:GFP<sup>lo</sup>) cells, c-myb expression in the CHT is increased in Rps14<sup>-/-</sup> compared to Rps14<sup>+/+</sup> (Figure 1M-O). Itga2b-GFP<sup>lo</sup>-expressing cells are some of the first to arise during definitive haematopoiesis and are restricted to HSPC and megakaryocytic lineage committed cells, while c-myb is expressed in a broader subset of HSPC as well as more committed myeloid lineage cells (http://servers.binf.ku.dk/bloodspot)<sup>27</sup>. Therefore this increase in expression in c-myb with normal itga2b and reduced mature myeloid cells suggests an increase in myeloid lineage restricted progenitor cells in Rps14<sup>-/-</sup> with a concomitant block in differentiation.

**Rps14<sup>-/-</sup> adult mutants have anaemia and features of MDS**

Rps14<sup>-/-</sup> adult zebrafish are significantly smaller than Rps14<sup>+/+</sup> (Figure 1P,Q, S4A,B). Haemoglobin levels showed a lower concentration of haemoglobin in Rps14<sup>-/-</sup> than Rps14<sup>+/+</sup> (Figure 1R). Furthermore, examination of haematopoietic cell morphology from Rps14<sup>-/-</sup> adults shows cells that have defective haemoglobinisation (Figure 1T,U, arrowhead) large red cells (Figure 1S) and dyserythropoiesis in the kidney marrow (Figures S4C-J).

We next used flow cytometric examination of kidney marrow (the site of adult haematopoiesis) to assess effects of Rps14 loss on haematopoiesis. At 5 months of age...
an increase in eosinophils and lymphocytes was observed in rps14+/− compared to rps14+/+ by forward and side scatter examination of cell populations (Figure 1V, S4K). We observed an increase in Tg(itga2b:GFPlo) cells within the progenitor cell fraction (Figure S4K,L), a feature observed in murine Rps14 conditional knockouts19,28. By 12 months the kidney marrow of Rps14+/− mutants shows defects in all lineages indicating features of bone marrow failure (Figure 1W, S4M).

**Stress markedly exacerbates the haematopoietic defects in Rps14 heterozygous animals.**

To refine the effects of Rps14 haploinsufficiency on haematopoiesis, we exposed Rps14+/− embryos to haematopoietic “stress”11. First, we used cold stress, incubating embryos at 22°C from 10hpf for 4 days to prim-22 (Figure S5A)29. Rps14+/− embryos were significantly more anaemic following cold stress than their Rps14+/+ siblings, with no clear difference in developmental stage (Figure S5B-D). To determine the effects of stress specifically on definitive haematopoietic cells, we used haemolytic stress induced by incubating embryos in PHZ for 24 hours (24hpf – 48hpf) and assessed recovery of haematopoiesis (Figure 2A)30. Embryos exposed to PHZ resulted in loss of haemoglobinised erythroid cells due to apoptosis (Figure S6). At 6dpf, WT animals had completely recovered, with o-dianisidine staining showing normal haemoglobinisation, while Rps14+/− siblings remained markedly anaemic (Figure 2B,C quantified in 2D). We further assessed the effect of haemolytic stress on HSPC using Tg(itga2b:GFP;rps14+/−). As previously, Rps14+/− embryos showed no difference in Tg(itga2b:GFP)-HSPC numbers in the absence of PHZ. PHZ stress resulted in an increase in HSPC compared to non-stressed animals in WT, however Rps14+/− larvae were unable to elicit this response leading to a significant decrease in HSPC compared to stressed WT siblings (Figure 2E).

**Small molecule screens identify imiquimod as a modifier of anaemia in Rps14-deficient embryos**

To utilise our system to assess for potential novel therapeutic agents in del(5q) MDS, we developed a small molecule screen for modifiers of anaemia (Figure 3A). We used MO knockdown of Rps14 as the morphant phenotype is comparable to mutants, allowing us to increase the number of molecules tested12. We screened the Spectrum collection ([http://www.msdiscovery.com/spectrum.html](http://www.msdiscovery.com/spectrum.html)) for compounds that could alleviate the characteristic morphological defects and/or anaemia resulting from Rps14 MO injection. Treatment of Rps14 morphants with the TLR7/8 pathway agonist imiquimod strikingly rescued the anaemia phenotype of Rps14 morphants (Figure 3D) compared to DMSO-
treated negative controls (Figure 3C) to a similar level observed in studies using L-Leucine\textsuperscript{12}.

We next assessed the improvement in haemoglobinisation with DMSO or 5\(\mu\)M imiquimod treatment by classifying each larva by the severity of their anaemia. Imiquimod markedly decreases the proportion of larvae with severe anaemia (Figure 3F).

**Imiquimod rescues stress-induced anaemia in Rps14 heterozygotes.**

We next assessed the effects of imiquimod on Rps14\textsuperscript{+/-} mutants exposed PHZ. As observed in Rps14 morphants, imiquimod rescued the anaemia observed in Rps14\textsuperscript{+/-}-stressed mutants (Figure 4A-E). We also observed a dose-dependent effect of imiquimod on the proportion of anaemic animals at 6 days (Figure 4F).

Inflammatory signalling through other TLRs has been shown to affect the emergence of HPSCs. To determine at which point during haematopoiesis imiquimod exerted its effect, and to assess the effect of TLR7 signalling on HSPC, we used Tg(itga2b:GFP);Rps14\textsuperscript{+/-} exposed to haemolytic stress and then treated with imiquimod or control. In PHZ stressed, control mutants, GFP\textsuperscript{lo} HSPC were reduced in Rps14\textsuperscript{+/-} compared to Rps14\textsuperscript{+/-}. Exposure to imiquimod resulted in an increase in GFP\textsuperscript{lo} cells in both Rps14\textsuperscript{+/-} and Rps14\textsuperscript{+/-}, however the effect was more marked in Rps14\textsuperscript{+/-} compared to Rps14\textsuperscript{+/-}. A 2-way ANOVA demonstrated an interaction between the effects of the Rps14 heterozygosity and imiquimod (Figure 4G).

These findings indicate that imiquimod leads to both improved haemoglobinisation and increased HSPC numbers.

**Imiquimod impacts erythropoiesis through TLR7 ligation**

Imiquimod is an imidazoquinoline, which binds to TLR7 and TLR8 on their dimerization interface. This binding is enhanced for TLR7 when ssRNA (its cognate ligand) is bound at a different site\textsuperscript{31,32,33,34}. To determine whether the haematopoietic effects of imiquimod we observed were through ligation of TLR7, we utilised additional small molecules that selectively activate these receptors. Gardiquimod, a specific TLR7 agonist rescued the anaemia associated with Rps14 heterozygosity (Figure 4H-L, N). In contrast we were unable to rescue the effects with the TLR8-specific agonist motolimod (Figure 4J, M). This indicates that the effects we observed were specific to ligation of TLR7 but not TLR8. To further confirm this we utilised a Tlr7 crispant knockout (Figure 4O,P). Rps14\textsuperscript{+/-} x Rps14\textsuperscript{+/-} clutches were injected with Tlr7 CRIPSR guide or control and then exposed to PHZ. TLR7
crispants abrogated the imiquimod-mediated rescue of anaemia in Rps14 heterozygotes (Figure 4Q). These results show that the effects of imiquimod observed occur specifically through TLR7.

**Imiquimod treated Rps14**+/− HSPC show reversal of WNT signalling and paradoxical downregulation of inflammation and an increase in erythroid differentiation.

We sought to define the mechanism by which “stress” from PHZ led to more marked anaemia in Rps14+/− and whether imiquimod was alleviating this to reverse the effects. We have previously observed an increase in phosphorylation of Eif2α at serine 51 (Eif2αP) in Rps19 morphants35, which occurs in response to stressors including free haem, resulting in global translation reduction. We hypothesised Eif2αP may also occur in Rps14−/− under haemolytic stress. At 6dpf Eif2αP:Total ratio was increased in Rps14−/− compared to Rps14+/+ (p=0.02), however this was not clearly influenced by the presence of PHZ or imiquimod (Figure 5A,B). Therefore we conclude that Rps14−/− results in Eif2αP independent of stress, which contributes to defective translation in our model.

To refine further the mechanism by which imiquimod increases HSPC and mature erythroid cells more potently in Rps14−/− than in WT, we performed RNASeq analysis of HSPC with or without PHZ stress treated with imiquimod or vehicle control (Figure S3C). Systems level analysis of genes confirm highly significant knockdown of Rps14 at the RNA level in HSPC in heterozygotes across all conditions (Figure 5C)36. Pathway analysis of genes differentially regulated in Rps14−/− compared to Rps14+/+ demonstrated enrichment of pathways involving ribosome biogenesis, translation, p53 and TNFalpha/NFκB signalling (Figure 5D), in keeping with known Rps14-associated pathways in haematopoiesis36,37. The number of differentially regulated genes was relatively few (35-225) between conditions, suggesting the observed phenotypes likely arise post-transcriptionally, or through non-cell autonomous effects. To further assess changes in differentially expressed genes we undertook Gene Set enrichment analysis (GSEA)38,39. We observed that negative regulators of canonical WNT signalling were upregulated in PHZ stressed Rps14−/− HSPC compared to siblings, but markedly downregulated following exposure to imiquimod (Figure 5E). We also observed reciprocal changes in inflammatory signalling signatures in stressed Rps14−/− compared to siblings, with exposure to imiquimod resulting in a change from pro- to anti-inflammatory signalling (Figure 5F). We validated this using qPCR for downstream mediators of TLR-signalling (Figure S7A). Consistent with our GSEA findings we showed that the presence of imiquimod reverses
the expression level of key NFκB pro-inflammatory target genes in Rps14 morphants. To further investigate these findings in Rps14/+− mutants we utilised a Tg(NFκB:GFP) reporter line. We found that PHZ stress led to an increased expression of GFP in Tg(NFκB:GFP) at 4dpf, but this effect was reversed in Rps14 +/- compared with Rps14+/- in the presence of imiquimod (Figure 5G). We also assessed levels of Il1/β using WISH. PHZ exposure resulted in an increased number of embryos with high levels of Il1/β expression, and this again was reversed in the presence of imiquimod (Figure 5H). Finally, we utilised a Myd88 MO to show that loss of Myd88 abrogated the imiquimod-mediated rescue of anaemia in Rps14+/- (Figure S7B), in a similar way to loss of Tlr7 (Figure 4Q). These data combined show that Rps14+/-, results in a pro-inflammatory state through activation of the TLR-MyD88-NFκB signalling complex, and treatment with imiquimod in Rps14+/- paradoxically reverses this effect.

Imiquimod enhances erythroid differentiation

Our data suggested that Rps14+/- treated with imiquimod resulted in an increase in HSPC but also an increase in more mature erythroid cells. To assess whether this was due to a direct effect on erythroid differentiation we assessed the differential effects of Rps14+/- with and without imiquimod on erythroid differentiation. GSEA showed that stressed HSPC have a downregulated erythroid differentiation signature in Rps14+/- compared to siblings. Imiquimod reverses this effect showing a pro-erythroid differentiation signature (Figure S8A,B). We used WISH to assess the expression of gata1 at 3dpf, and showed that during recovery from PHZ stress there is an increase in gata1 expression which is more pronounced in Rps14+/- compared with Rps14+/- and that this effect is reduced in the presence of imiquimod (Figure S8C). To further assess these effects, we ablated all mature erythroid cells using MO knockdown of master erythropoiesis regulator Gata1 in Tg(gata1:dsRed) transgenic animals40,41. GATA1 has been shown as central to the mechanism of anaemia associated with RPS14 and other ribosomal proteins42-47. Treatment of Gata1 morphants with imiquimod increased the numbers of circulating and static erythroid cells (Figure S8E-I and supplemental movies), and cytospin of sorted dsRed cells showed increased numbers of mature erythroid and myeloid cells (Figure S8J-L). Therefore our data support that imiquimod can not only increase HSPC numbers but promote both erythroid and myeloid differentiation on the background of anaemia associated with Rps14 or Gata1 deficiency.

In vitro haematopoietic colony output is enhanced in anaemic human primary cells treated with imiquimod
Our zebrafish studies show that imiquimod increases HSPC and can alleviate anaemia by enhancing erythroid differentiation in Rps14- and Gata1-deficient anaemia. To determine if these effects were also observed in human cells we sought to analyse patient bone marrow (BM) with MDS. Unfortunately there were no patient samples with MDS del (5q) available for analysis. However, we think that the effect of imiquimod is likely not limited to patients with ribosomal protein abnormalities but more, those who have a basal inflammatory state which is common to loss of Rps14 and other MDS subtypes. Therefore we utilized samples from anaemic patients dysplasia in their bone marrow (Table S1). HSPC output from patients with anaemia was extremely low with less than 0.02% of plated cells giving rise to any colonies (Figure 6A). This contrasts with the non-anaemic control where 8% of plated cells gave rise to colonies (Figure S9A) and where the overall colony output was enhanced by imiquimod.

In both CMPs and MEPs from anaemic patients we observed an increase in colony output in the imiquimod-treated cells compared to controls (Figures 6B and 6C). This reflects an increase in both colony forming units - granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) and burst forming unit-erythroid (BFU-E). In contrast there was no effect on overall colony output from GMPs, rather a lineage bias towards more mature myeloid (GM/G and M) colonies rather than GEMM (Figure 6D). To further highlight the effects at the MEP and CMP level, Figures 6E-J show the output from each patient with or without the addition of imiquimod. Whilst these results did not reach significance, taken together these data indicate that the effects of imiquimod treatment on erythropoiesis may result from effects both at the level of CMPs and MEPs in human cells. Our data also highlight that imiquimod has variable effects at different stages of lineage commitment in both the myeloid and erythroid compartment and these are not limited to Rps14 deficiency, suggesting crosstalk between mediators of inflammation may occur more widely.

Discussion

In this study we have generated a model of del(5q) MDS using TALENS to introduce a frameshift mutation in Rps14. Embryos deficient in Rps14 show dose-dependent defects in mature myeloid cell numbers. Erythropoiesis is similarly reduced, however the effects on the erythroid lineage as well as HSPC are much more striking following exposure to stress. These findings suggest that Rps14 haploinsufficiency has significant effects on steady state haematopoiesis but that Rps14 also has a specific role in maintenance of stress-induced haematopoiesis. As PHZ results in stress to the haematopoietic system by...
haemolysis, we assessed whether Rps14-specific effects of PHZ may be mediated by stress-response pathways sensitive to free haem via Eif2\(\alpha\)P. Interestingly, although we did not show an effect of PHZ on Eif2\(\alpha\)P, Rps14\(^{-/-}\) mutants regardless of treatment showed an increase in Eif2\(\alpha\)P compared to siblings. This suggests that a global reduction in translation arising from Eif2\(\alpha\)P may contribute to the phenotypes observed.

To identify potential novel therapeutic agents for del(5q) MDS, we conducted an in vivo small molecule screen for compounds that could alleviate anaemia in Rps14-deficient embryos. This screen identified a striking rescue of the anaemic phenotype with the TLR7/8 agonist imiquimod. Our data showed imiquimod exerts its effect on erythroid cells via TLR7 signalling. We also showed a marked effect of imiquimod on itga2b:GFP-expressing HPSC. Effects were observed in Rps14\(^{-/-}\) as well as Rps14\(^{+/+}\), but notably the effect in Rps14\(^{-/-}\) was more pronounced.

Pro-inflammatory signalling through TLR4 has been identified as a mechanism of anaemia in Rps14-deficient mice, and blocking this signalling pathway can rescue this effect\(^{11}\). Rps14\(^{-/-}\) HSPC showed upregulation of TNF/NF\(\kappa\)B signalling complex in our model indicating conservation of this pathway. However TLR7 ligation also activates this pathway through Myd88. Therefore our findings that activation of this pathway alleviates anaemia, appear counterintuitive. RNAseq data support that TLR7 activation in Rps14\(^{-/-}\) HSPC paradoxically results in downregulation of inflammation, which we validated by measuring NF\(\kappa\)B and Il1\(\beta\) expression levels. A paradigm for such crosstalk between activation of different TLRs and the effects of this on downstream signalling and homeostasis of inflammatory signalling has previously been elucidated for TLR3 and TLR7\(^{50}\). Co-activation of TLR3 and TLR7 leads to an enhanced production of some inflammatory cytokines, however, production of a number of key pathway intermediates such as TRAF6 is reduced. Induction of inflammatory tolerance has also been described between TLR4 and TLR7/8 in monocytes which required microRNA 146a\(^{51}\). This suggests that while some components of the innate immune response act in concert to enhance inflammatory signalling, there are underlying mechanisms in place to halt excessive immune activation, or even specifically reduce inflammation.

The effects of imiquimod in our model were not limited to increased HSPC numbers. Using the differentiation arrest observed in Gata1 morphants, were demonstrated an effect of imiquimod on erythroid (as well as myeloid) differentiation. Interestingly Gata1 not only has a central role in the pathogenesis of ribosomal protein-mediated anaemias, it is also thought to be key in cytopenias associated with activation of the inflammasome in general.
When comparing Rps14\(^{+/−}\)-stressed HSPC with siblings, stressed HSPC show a significant increase in negative regulators of the canonical WNT pathway. Regulation of HSPC specification, emergence, expansion and differentiation have all been shown to be tightly regulated, in part, by components of the WNT/β-catenin pathway\(^{52,53}\). However, temporally, both imiquimod treatment and the effects we observe occur after specification and emergence of HSPC, suggesting in this context WNT pathway inhibition in stressed Rps14\(^{+/−}\) may impede expansion or differentiation of HSPC, and that this is alleviated by imiquimod. Furthermore crosstalk between NFκB inflammatory signalling and WNT pathway activation and/or inhibition has been reported in a number of cell types (reviewed in \(^{54}\)), including evidence that prolonged TLR4-mediated inflammation suppresses WNT/β-catenin in bone resulting in apoptosis and necrosis\(^{55}\). Our study highlights possible crosstalk between these pathways may occur.

Although, TLRs, their ligands and the inflammatory consequences of signalling have been shown to differ between species\(^{56}\). TLR7 and TLR8 are the most highly conserved of all the TLRs and inflammatory responses to R848 are preserved in fish\(^{57}\). Nonetheless, to establish the relevance of our findings for humans, we utilised bone marrow cells from anaemic patients to determine the effects of imiquimod. Our findings suggest that imiquimod can enhance erythroid output in anaemic individuals at the level of both CMP and MEP, supporting the data from the fish. In these experiments, very few colonies were derived from HSC of anaemic patients therefore assessment of the effects of imiquimod on HSC was not possible. One possibility is that the effects observed in our system are non-cell autonomous via other inflammatory cells. The majority of functional effects of TLR7 signalling have been described via innate immune cells. Furthermore, we observed a larger inflammatory signalling response when analysing downstream effector genes of TLR signalling in whole embryo RNA extracts (Figure 5, S7). Thus the cell of origin of the effects observed in our studies is not yet defined and is the focus of ongoing work.

Finally, one key unanswered question is how Rps14 deficiency and associated effects on ribosome assembly and translation leads to activation of inflammatory signalling. Murine studies define increased production of S100 proteins to be the mediators of increased inflammatory signalling via TLR4\(^{11}\). However, it is attractive to speculate, given the structural knowledge of TLR7 activation by imiquimod being enhanced by ssRNA species, that the production of aberrant rRNA species observed in Rps14 knockout cells may contribute to our observation that the effects of imiquimod are more marked in Rps14-deficient cells\(^{5,58}\). To date, no such phenomenon has been observed for endogenous RNA
species outside of autoimmune disease.

In summary, we describe a model of del(5q) MDS in zebrafish and define a novel role for the TLR7 in enhancing haematopoiesis in this model through modulation of the inflammatory response and differentiation.

Data Sharing Statement

Sequencing files have been deposited in GEO under the number GSE168727. All other data is available on request from the corresponding author

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Authorship Contributions

EP, OP and AL designed and carried out experiments. JR, CH, AL, PD and YH carried out experiments and contributed to scientific analysis and discussion. YJ conducted the screen with assistance from MV and OP. LV and KT made TALENS targeting rps14. CB and SR provided essential technical assistance for human haematopoietic cell studies and analysis.

Conflict-of-interest disclosure

E.P has received honoraria for consultancy for Novartis, Celgene and Takeda not related to the work in this manuscript.
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Figure Legends

Figure 1. Rps14 stable mutants show dose-dependent effects on haematopoiesis in embryos. (A-C) Assessment of haemoglobinised cells using α-dianisidine staining. Ventral views of the head in 4dpf embryos (A-C). Rps14<sup>−/−</sup> zebrafish show profound loss of haemoglobinised cells (C) and developmental anomalies. Rps14<sup>+/−</sup> are indistinguishable from their WT siblings by microscopy (A, B). (D) Erythroid cell number by flow cytometry of individual Tg(gata1:dsRed);Rps14<sup>+/−</sup> embryos at 3dpf. There is an allelic dose-dependent effect on dsRed-expressing cell number. (E-G) 4dpf SB-stained embryos to stain granulocytes. SB positive cell number quantified in (H). Region of CHT depicted in cartoon (cartoon adapted from Lizzy Griffiths with permission) (K). SB staining shows an allelic dose-dependent effect of rps14. (I,J) Lateral views of the CHT of 4dpf Rps14 mutant fish carrying itga2b:GFP transgene, labelling HSPC that reside in the CHT. (L) shows quantitation of stationary GFP<sup>lo</sup> cells in the CHT. (M,N) Expression of c-myb by in situ hybridisation in 4dpf embryos. In contrast to itga2b-GFP, c-myb expression is increased in the CHT, quantified by median expression intensity in CHT (O) (P,Q) Lateral views of 12-month-old adult fish show a decreased size of heterozygotes. Anterior is shown to the left and dorsal upwards. Line shows the body length excluding the tail (R) shows quantification of haemoglobin concentration. (S) Shows analysis of cell size, red blood cells are significantly larger cells in Rps14<sup>+/−</sup> mutants compared to siblings (T,U) show blood smears from Rps14<sup>+/−</sup> and WT siblings, red arrowhead highlighting poorly haemoglobinized erythroid cell in rps14<sup>+/−</sup>. (V) and (W) absolute cell number per microliter of different cell types in the kidney marrow of 5 and 12 months old fish respectively, showing progressive differences between heterozygous rps14 mutants and their wild type siblings.

Statistical comparison by one-way ANOVA with Tukey’s multiple comparisons test (D,H) or unpaired t-tests.

Figure 2. Haemolytic stress augments the haematopoietic phenotype in Rps14<sup>+/−</sup> mutants, and this is rescued by imiquimod (A) Schematic of haemolytic stress experiment (B,C) Representative views (ventral) of rps14<sup>+/+</sup> and rps14<sup>+/−</sup> siblings treated as in (A) and stained with α-dianisidine at 6dpf. Rps14<sup>+/−</sup> demonstrate a clear anaemic phenotype, quantified in (D). (E) effect of haemolytic stress on HSPC. Statistical comparisons were carried using Fisher’s exact test (D) or ANOVA (E).

Figure 3. Small molecule screen for modifiers of anaemia in Rps14 deficiency
identifies imiquimod. (A) Schematic of the screen design. (B-E) 4dpf rps14 morphants and controls treated with DMSO, L-leucine or imiquimod stained for haemoglobin with α-dianisidine. Upper panels shows ventral views of the head, lower panels show lateral views, with anterior to the left and dorsal upwards. (F) semiquantitative analysis of the effects of imiquimod on haemoglobinization severity. Imiquimod improves the level of haemoglobinization compared to DMSO treated controls. Statistical comparisons were carried out by Fisher’s exact test (F).

Figure 4. Imiquimod exerts its effect on Rps14-deficient anaemic embryos via on target activation of TLR7 (A-D) Ventral views of 6dpf embryos stained with α-dianisidine, treated haemolytic stress and DMSO (A and C) or imiquimod (B and D). Imiquimod rescues stress induced anaemia. Quantified as ratio of normal:total embryos across 3 experiments for 20µM (F). Effect of imiquimod analysed across dose range using non-linear regression (E). (G) Flow cytometric analysis of $Rps14^{E8fs}, Tg(itga2b:GFP)$ single embryos exposed to haemolytic stress and then treated with DMSO or imiquimod. Imiquimod enhances $itga2b:GFP^b$ cells and this is most marked in Rps14$^{+/s}$ where there is a significant interaction between the drug and genotype. (H-M) Ventral views of 6dpf embryos stained with α-dianisidine, treated haemolytic stress and DMSO (H and K) or gardiquimod (I and L) or motolimod (J and M). Gardiquimod but not motolimod rescues the stress-induced anaemia in $Rps14^{+/s}$ embryos. Gardiquimod rescue effect analysed across dose range using non-linear regression (N). Tlr7 knockout validated using Miseq (O) and restriction enzyme digest with BplI which digests only the WT (P). (Q) Knockout of Tlr7 abrogates rescue of anaemia by imiquimod.

Figure 5. RNASeq analysis of HSPC show effects on WNT and inflammatory signalling pathways. (A) Representative western blot of p-Eif2α (phosphoserine 51) and Eif2α total in Rp14 mutants at 6dpf exposed to PHZ and/or imiquimod (B) Normalised ratio (to untreated WT) of p-Eif2α:Total Eif2α shown for 3 experiments represented by different coloured lines. p-value refers to the effect of genotype on the ratio. (C) differential gene expression of Rps14$^{+/s}$ vs Rps14$^{+/s}$ shown as a volcano plot for all conditions combined. Rps14 is highlighted in bold

(D) Metascape pathway analysis of Rps14$^{+/s}$ vs Rps14$^{+/s}$ differentially expressed genes showing top 20 enriched pathways. (E,F) GSEA analysis comparing Rps14$^{+/s}$ vs Rps14$^{+/s}$ in DMSO treated vs imiquimod treated HSPC. Negative regulation of WNT signalling is enriched in Rps14$^{+/s}$ vs Rps14$^{+/s}$ DMSO treated HSPC and this is reversed imiquimod treated HSPC (E). Similarly inflammatory signalling is enriched in Rps14$^{+/s}$ vs Rps14$^{+/s}$ DMSO treated HSPC but suppressed in imiquimod-treated HSPC (F). (G) Quantification of
total fluorescence of NFKβ:GFP at 4dpf shows an increase with PHZ stress but a
decrease with imiquimod in Rps14+/− compared with Rps14+/+. (H) PHZ causes an
increase in il1β expression by in situ hybridization in Rps14+/− which is rescued by
imiquimod. Statistical comparisons carried out with 2-way ANOVA (B,H genotype and
condition) or ANOVA (G).

Figure 6. Imiquimod enhances erythroid output of human primary cells in vitro. (A-
D) Primary cells obtained from bone marrows of anaemic patients detailed in Table S1
(n=4) were FACS-sorted into different populations. Sort purity was verified as greater than
95% for all conditions. CFU-assays were carried out in triplicate in methocult without
serum. Colonies were scored at 12 days and output is shown as percentages of cells
input. HSPC (A), CMPs (B), MEPs (C), and GMPs (D). (E-G) Individual patient plots
highlighting the change in colony output associated with imiquimod for MEPs (E-H) and
CMPs (I,J). HPSC, haematopoietic stem cells; CMP, common myeloid progenitor; MEP,
megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor; BFUE, burst
forming unit erythroid; GEMM, granulocyte, erythrocyte, macrophage and megakaryocyte;
GM/G, granulocyte, macrophage / macrophage; M, macrophage. AML_PR, acute myeloid leukaemia in partial remission;
MDS- MD, myelodysplastic syndrome with mutli-lineage dysplasia; DLBCL, diffuse large B
cell lymphoma.
**Figure 1**

### 4dpf

- **A**: rps14
- **B**: rps14
- **C**: rps14
- **D**: rps14

### 3dpf

- **H**: SB positive cells
- **L**: Area of cell x 10 pixels

### 5 months

- **R**: Haemoglobin (g/L)
- **S**: Area of cell x 10 pixels

### 12 months

- **V**: Blood
- **W**: 12 months
Figure 2

O-dianisidine 6dpf; 4 days post PHZ

A

B

C

D

E

Anaemia level

HSPC numbers 4dpf

Percentage of embryos

p = 0.009

Rps14+/+

Rps14-/

Rps14+/+

Rps14-/

p = 0.0003

p = 0.0005

itga2b::GFP cells / CHT

PHZ

- - + +

ns

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A

Inject rps14 MO

Primary Screen

Secondary Screen

Tertiary Screen

Spectrum Library

5μM and 20μM

α-Dianisidine staining

Fresh compound

Range of doses

2 observers

B C D E

uninjected DMSO

rps14 MO DMSO

rps14 MO 20μM imiquimod

rps14 MO 100mM L-leucine

4dpf

F

MO drug control control rps14 rps14

drug DMSO imiquimod DMSO imiquimod

p=0.0079
