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STAT3 mutations are not sufficient to induce large granular lymphocytic leukaemia in mice

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Keywords

STAT3; large granular lymphocytic leukaemia; mouse model

Large granular lymphocytic (LGL) leukaemia is a rare lymphoproliferative disorder characterized by the expansion of $CD3^+CD8^+$ cytotoxic T-lymphocytes (CTLs) or $CD3^-$ natural killer (NK) cells. LGL leukaemia can be subdivided into T-cell LGL (T-LGL) and chronic lymphoproliferative disorders of NK cells (CLPD-NK) depending on the type of cells affected. Patients with LGL leukaemia exhibit anaemia, neutropenia, splenomegaly and autoimmune disorders (Steinway *et al,* 2014). Somatic mutations in the signal transducer and activator of transcription 3 gene (*STAT3*) have been found in 40% of T-LGL and 30% of CLPD-NK patients. These *STAT3* mutations result in constitutive activation of STAT3 protein. The discovery of *STAT3* mutations in T-LGL and CLPD-NK has suggested a role for *STAT3* mutations in the pathogenesis of these diseases (Koskela *et al,* 2012; Jerez *et al,* 2012). However, it remains still unclear whether *STAT3* mutations play a causal role in the development of LGL leukaemia.

To investigate the effects of *STAT3* mutations on haematopoietic cells, murine interleukin 3 (IL3) -dependent Ba/F3 cells were transduced with MSCV-IRES-GFP retrovirus expressing empty vector, wild-type STAT3 ($STAT3^{WT}$) or LGL leukaemia-associated STAT3 mutants ($STAT3^{D661V}$, $STAT3^{Y640F}$), and the infected cells were sorted for GFP. Immunoblot analysis showed increased phosphorylation of STAT3 in Ba/F3 cells expressing $STAT3^{D661V}$ or $STAT3^{Y640F}$ mutant compared to vector or $STAT3^{WT}$ (Fig.S1A). However, expression of $STAT3^{D661V}$ or $STAT3^{Y640F}$ mutant did not provide any growth advantage to Ba/F3 cells when compared with vector or $STAT3^{WT}$ expression (Fig.S1B).

To assess the *in vivo* effects of *STAT3* mutations on haematopoiesis, we performed retroviral bone marrow (BM) transduction and transplantation assays as outlined in Fig.1B.

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Authorship contributions

A.D. performed research, analysed data and wrote the manuscript; D.Y. performed research; R.E.H. conducted histopathological analysis and revised the manuscript; G.M. designed the research, analysed data, and wrote the manuscript.

Conflict of interest disclosure

The authors declare no conflict of interest.

Expression of $STAT3^{D661V}$ or $STAT3^{Y640F}$ resulted in increased phosphorylation of STAT3 in 293T cells (Fig.1A). Transplanted animals expressing $STAT3^{Y640F}$ exhibited significantly increased neutrophil counts compared with mice expressing $STAT3^{WT}$ at 16 weeks after transplantation (Fig.1C). Also, there was trend of increased neutrophil counts in the transplanted animals expressing $STAT3^{D661V}$ (Fig.1C). No significant difference was observed in white blood cell, lymphocyte, red blood cell and platelet counts or haematocrit among transplanted animals expressing $STAT3^{WT}$, $STAT3^{D661V}$ or $STAT3^{Y640F}$ (Fig.1C). Expression of $STAT3^{Y640F}$ resulted in modest but significant increase in spleen size/weight in the transplanted mice whereas $STAT3^{D661V}$ mice had slightly larger spleen than that of $STAT3^{WT}$ mice (Fig.1D). Histopathological analyses of the BM sections showed increased granulocytic precursors in $STAT3^{Y640F}$ mutant mice, whereas $STAT3^{D661V}$ mutant mice exhibited mildly increased myeloid cells compared with the BM of $STAT3^{WT}$ mice (Fig.1E). However, there were no increases in LGL cells in the BM of $STAT3^{D661V}$ or $STAT3^{Y640F}$ mutant mice. Spleen sections from both $STAT3^{Y640F}$ and $STAT3^{D661V}$ mice showed minimal increase in myeloid cells compared to $STAT3^{WT}$ mice (Fig.1E). Flow cytometric analysis showed modest increases in myeloid ($Gr\text{-}1^+/Mac\text{-}1^+$) cells in the BM of mice expressing $STAT3^{Y640F}$ compared with $STAT3^{WT}$-expressing mice (Fig.2A, B and Fig.S2). However, we did not see any significant alterations in the erythroid ($Ter119^+/CD71^+$), megakaryocytic ($CD41^+/CD61^+$), B-cell ($B220^+$) and T-cell ($Thy\text{-}1^+$) precursors in the BM and spleens of $STAT3^{D661V}$ or $STAT3^{Y640F}$ mice compared with $STAT3^{WT}$ mice (Fig.2A, B and Fig.S2). As T-LGL is characterized by an expansion of $CD3^+CD8^+$ CTLs (Koskela *et al,* 2012), we also determined the $CD3^+CD8^+$ populations in the peripheral blood (PB), BM and spleens of the transplanted animals expressing $STAT3^{WT}$, $STAT3^{D661V}$ or $STAT3^{Y640F}$. We observed no significant difference in $CD3^+CD8^+$ populations in the PB, BM and spleens of $STAT3^{D661V}$ or $STAT3^{Y640F}$ mutant mice compared with $STAT3^{WT}$ mice (Fig.2C). We also did not observe any significant difference in $CD3^-$ natural killer cells ($CD3^-NK1.1^+$) in the BM and spleens of the $STAT3^{D661V}$ or $STAT3^{Y640F}$mutant mice compared with $STAT3^{WT}$ mice (Fig.2D). The frequency of $CD3^-NK1.1^+$ cells was rather decreased in the PB of $STAT3^{D661V}$ or $STAT3^{Y640F}$ mutant mice compared with $STAT3^{WT}$ mice (Fig.2D). The frequency of $CD3^+CD8^+$ and $CD3\text{-}NK1.1^+$ cells in the thymus was comparable in $STAT3^{WT}$, $STAT3^{D661V}$ and $STAT3^{Y640F}$ mice (Fig.S3A, B). We also assessed the engraftment of haematopoietic cells in the BM of the transplanted animals using GFP expression. As shown in Figure 2E, a significant proportion of cells (~40%) in the BM of transplanted mice receiving $STAT3^{D661V}$ or $STAT3^{Y640F}$-transduced BM were GFP^+, suggesting that donor BM cells were efficiently engrafted. Immunoblot analysis showed constitutive phosphorylation of STAT3 in mice expressing $STAT3^{D661V}$ or $STAT3^{Y640F}$mutants (Fig.2F), confirming that the STAT3 mutants were efficiently expressed in the BM of the transplanted animals. Together, these data suggest that *STAT3* mutations associated with LGL leukaemia results in constitutive activation of STAT3; however, expression of *STAT3* mutant alone is not sufficient to induce LGL leukaemia in a mouse model. These data are in accordance with a previous report (Couronné *et al,* 2013) showing that expression of $STAT3^{Y640F}$ mutation primarily induces myeloid malignancy in a murine BM transplantation model.

STAT3 can be activated by oncogenic tyrosine kinases (Vainchenker & Constantinescu 2013), by autocrine production of IL6 (Schuringa *et al*, 2000) or through somatic *STAT3* mutations (Koskela *et al,* 2012; Jerez *et al,* 2012). Constitutive phosphorylation/activation of STAT3 has been observed in various human malignancies (Yu *et al,* 2014). The identification of *STAT3* mutations in significant cases of T-LGL and CLPD-NK has suggested the use of STAT3 inhibitors as potential treatment for LGL leukaemia (Koskela *et al,* 2012; Jerez *et al,* 2012). If *STAT3* mutations are not the driver of LGL leukaemia, therapies targeting STAT3 might not be very effective against LGL leukaemia. The absence of any LGL leukaemia phenotype in mice expressing STAT3 mutations implies that additional gene mutations or deregulation of other signalling molecules or pathways might be involved in association with *STAT3* mutations in the pathogenesis of LGL leukaemia. Somatic mutations in *STAT5B*, *PTPRT*, *BCL11B*, *SLIT2* and *NRP1* genes affecting the STAT pathway and/or T-cell activation also have been found at a lower frequency in LGL leukaemia (Rajala *et al,* 2013; Andersson *et al,* 2013). Moreover, decreased SOCS3 expression and increased plasma levels of IL6 have been observed in patients with LGL leukaemia (Teramo *et al,* 2013). Future studies will identify co-existing mutations or genetic abnormalities in LGL leukaemia and determine the cooperative effects of such genetic abnormalities along with *STAT3* mutations in the pathogenesis of LGL leukaemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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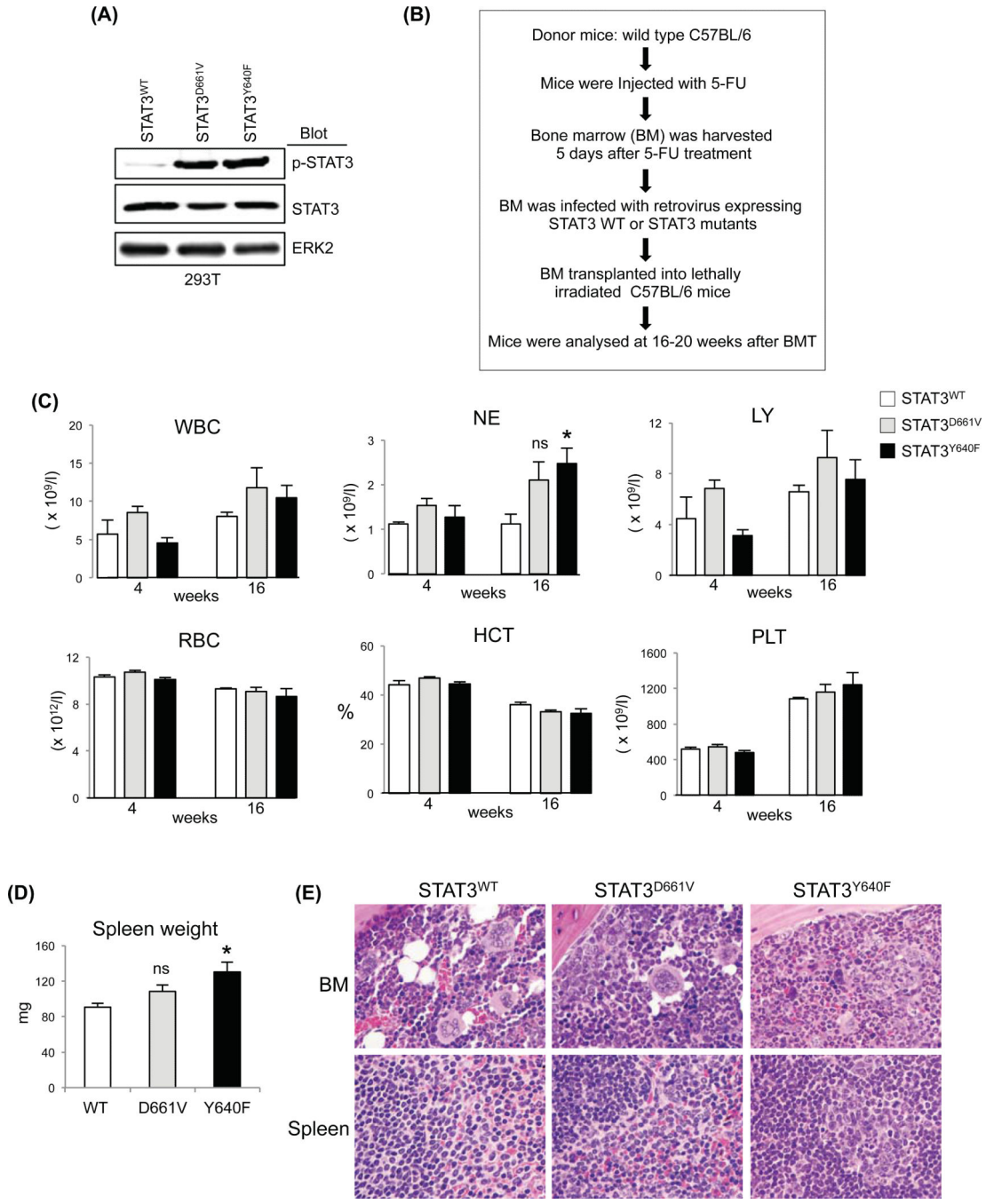

Fig 1.

Analysis of the *in vivo* effects of *STAT3* mutations in a mouse bone marrow transplantation model. (A) Retroviruses expressing $STAT3^{WT}$, $STAT3^{D661V}$ or $STAT3^{Y640F}$ were produced in 293T cells. Immunoblot analysis show that expression of $STAT3^{D661V}$ or $STAT3^{Y640F}$ mutant resulted in increased phosphorylation of STAT3 in 293T cells. ERK2 was used as a loading control. (B) Experimental design on retroviral bone marrow (BM) transduction/ transplantation assay. Wild-type C57BL/6 mice were injected with 5-fluorouracil (5-FU) 5 days prior to BM harvest. BM cells were transduced with retroviruses expressing $STAT3^{WT}$,

$STAT3^{D661V}$ and $STAT3^{Y640F}$ and the infected cells were transplanted into lethally irradiated C57BL/6 recipient mice (10^6 cells/recipient) by retro-orbital injection. Mice were analysed between 16-20 weeks after bone marrow transplantation (BMT). (C) Peripheral blood white blood cell (WBC), neutrophil (NE), lymphocyte (LY), red blood cell (RBC) and platelet (PLT) counts, and haematocrit (HCT) were assessed in the recipient mice expressing $STAT3^{WT}$ (n=4), $STAT3^{D661V}$ (n=4) and $STAT3^{Y640F}$ (n=4) at 4 and 16 weeks after BMT. (D) Spleen weights of transplanted animals expressing $STAT3^{WT}$, $STAT3^{D661V}$ and $STAT3^{Y640F}$ are presented in bar graphs as mean ± SEM (* indicates $p<0.05$ by Student's *t*-test; ns indicates not significant). (E) Histopathological analyses of the haematoxylin and eosin (H&E) stained BM sections showed increased granulocytic precursors in transplanted animals expressing $STAT3^{Y640F}$ mutant, whereas BM of transplanted animals expressing $STAT3^{D661V}$ mutant exhibited mildly increased granulocytic cells compared with the BM of $STAT3^{WT}$-expressing mice. Spleen sections from transplanted animals expressing $STAT3^{D661V}$ and $STAT3^{Y640F}$ showed minimal increase in myeloid cells compared to spleens of control $STAT3^{WT}$-expressing mice.

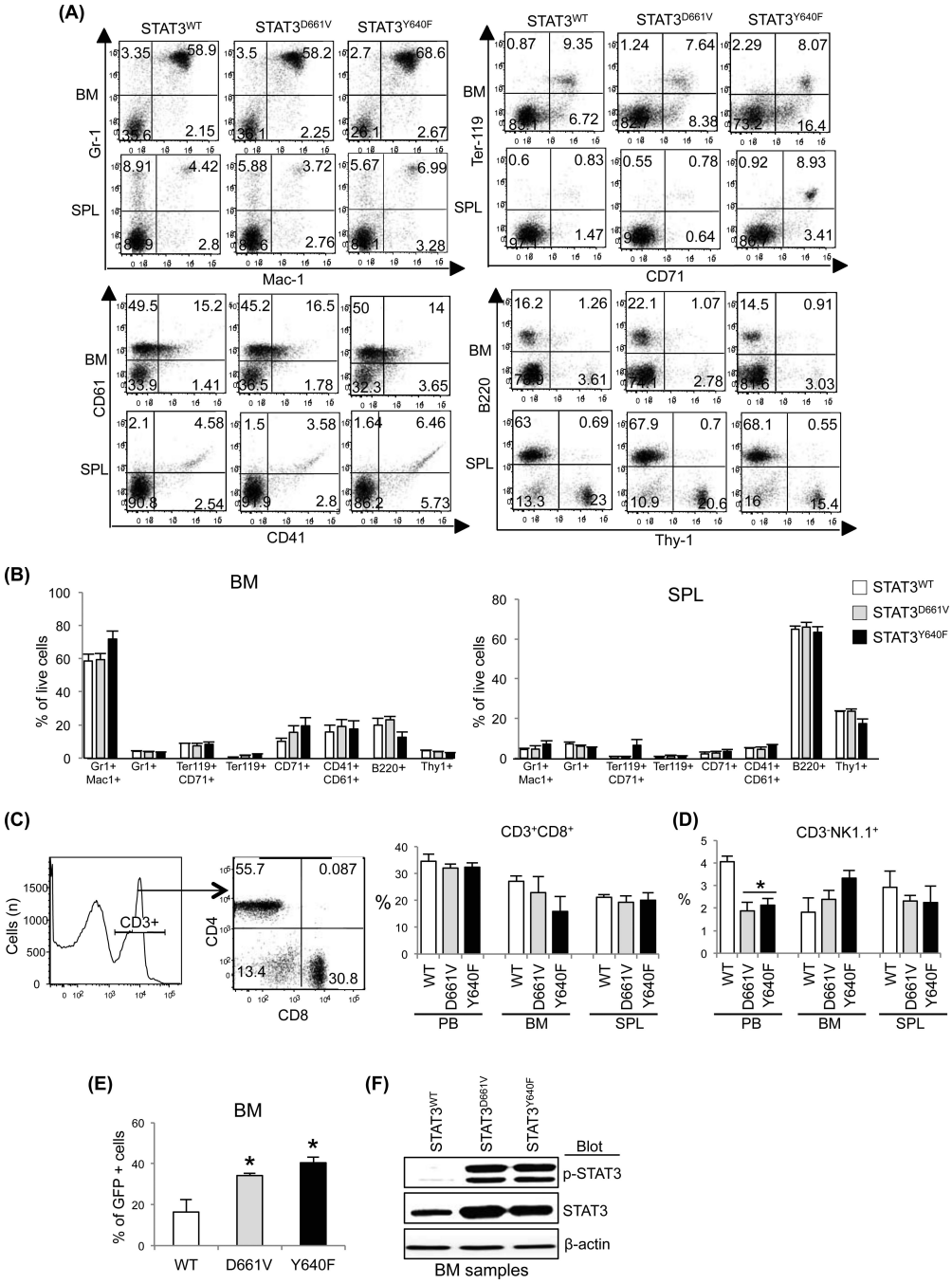

Fig 2.

Effects of *STAT3* mutations on haematopoietic precursors. (A) Representative dot plots of flow cytometric analysis of myeloid (Gr-1$^+$/Mac-1$^+$), erythroid (Ter119$^+$/CD71$^+$), megakaryocytic (CD61$^+$/CD41$^+$), B-cell (B200$^+$) and T-cell (Thy-1$^+$) precursors in the bone marrow (BM) and spleens of transplanted animals expressing STAT3^{WT}, STAT3^{D661V} and STAT3^{Y640F} at 16-20 weeks after bone marrow transplantation (BMT) are shown. (B) Percentages of myeloid (Gr-1$^+$/Mac-1$^+$), erythroid (Ter119$^+$/CD71$^+$), megakaryocytic (CD61$^+$/CD41$^+$), B-cell (B200$^+$) and T-cell (Thy-1$^+$) precursors in the BM and spleens

(SPL) of transplanted animals expressing $STAT3^{WT}$ (n=4), $STAT3^{D661V}$ (n=4) and $STAT3^{Y640F}$ (n=4) are shown in bar graphs as mean ± SEM. (C) Flow cytometric analysis of $CD3^{+}CD8^{+}$ cells in the peripheral blood (PB), BM and spleens of transplanted animals expressing $STAT3^{WT}$, $STAT3^{D661V}$ and $STAT3^{Y640F}$ at 16-20 weeks after BMT. (D) Percentages of $CD3^{-}$ natural killer cells ($CD3^{-}NK1.1^{+}$) in the peripheral blood (PB), BM and spleens of transplanted animals expressing $STAT3^{WT}$, $STAT3^{D661V}$ and $STAT3^{Y640F}$ at 16-20 weeks after BMT are shown in histograms as mean ± SEM (n=4). (E) Flow cytometric analysis of GFP expression in the BM at 16-20 weeks after BMT. Data are shown in histograms as mean ± SEM (n=4; * indicates $p<0.05$ by Student's *t*-test). Note that significantly increased percentages of GFP^{+} cells in the BM of transplanted animals expressing $STAT3^{D661V}$ or $STAT3^{Y640F}$ compared with transplanted animals expressing $STAT3^{WT}$. (F) Immunoblot analysis on total BM extracts shows constitutive phosphorylation of STAT3 in the BM of transplanted animals expressing $STAT3^{D661V}$ and $STAT3^{Y640F}$ mutants, suggesting that the STAT3 mutants were efficiently expressed and constitutively activated in the BM of the transplanted animals. β-actin was used as a loading control.