Interleukin-1 receptor antagonism leads to improved anaemia in a murine model of sickle cell disease and is associated with reduced *ex vivo* platelet-mediated erythrocyte sickling

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Summary

Sickle cell disease (SCD) is associated with haemolytic anaemia and secondary activation of leucocytes and platelets, which in turn may further exacerbate haemolysis. As cytokine signalling pathways may participate in this cycle, the present study investigated whether pharmacological blockade of the interleukin-1 receptor (IL-1R) would mitigate anaemia in a murine model of SCD. Within 2 weeks of treatment, reduced markers of haemolysis were observed in anakinra-treated mice compared to vehicle-treated mice. After 4 weeks of anakinra treatment, mice showed increased numbers of erythrocytes, haemoglobin, and haematocrit, along with reduced reticulocytes. Blood from anakinra-treated mice was less susceptible to *ex vivo* erythrocyte sickling and was resistant to exogenous IL-1β-mediated sickling. Supernatant generated from IL-1β-treated platelets was sufficient to promote erythrocyte sickling, an effect not observed with platelet supernatant generated from IL-1R$^{-/-}$ mice. The sickling effect of IL-1β-treated platelet supernatant was inhibited by a transforming growth factor-β (TGF-β) neutralising antibody, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibition, and superoxide scavengers, but replicated by recombinant TGF-β. In conclusion, pharmacological IL-1R antagonism leads to improved anaemia in a murine SCD model. IL-1β stimulation of platelets promotes erythrocyte sickling. This effect may be mediated by platelet-derived TGF-β-induced reactive oxygen species generation though erythrocyte NADPH oxidase.

Keywords: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, oxygen radicals, platelets, sickle cell disease, cytokines.

Introduction

Haemolytic anaemia in sickle cell disease (SCD) is associated with the activation of inflammatory pathways that may play a role in SCD-related complications, including the severity of anaemia.[1] Multiple cytokines have been shown to be elevated in SCD and to correlate with disease severity.[1] Toll-like receptors (TLRs) and NLR family pyrin domain containing 3 (NLRP3) inflammasome expression levels, including interleukin-1β (IL-1β), are increased in patients with SCD.[2–4] IL-1β is a particularly important mediator of haemolysis-related inflammation, which may promote vaso-occlusion.[1,5] While these previous studies suggest IL-1β signalling pathways are involved in some manifestations of SCD, the causal role of these pathways remains unclear.

In a chimeric model of SCD generated by bone marrow transplantation of sickle cell bone marrow into interleukin-1 receptor (IL-1R) deficient recipients, we have previously demonstrated that non-haematopoietic cellular IL-1R pools provide protection against organ damage and stroke.[6] These effects appear to be mediated via reduced endothelial adhesive properties, limiting leucocyte extravasation at sites of injury in SCD mice. However, these experiments did not assess the effects of IL-1R signalling on haematopoietic cells due to the nature of the chimeric mice generated by bone marrow transplantation. Protective effects of IL-1R deficiency involving the haematopoietic cellular pool may also be relevant in SCD. The therapeutic targeting of IL-1β has proven beneficial in several inflammatory diseases.[7–9] Pharmacological inhibition of IL-1R with the IL-1R1 antagonist protein,

anakinra, was shown to prevent the increased stroke size in SCD mice when given as a single dose shortly after stroke induction,[6] supporting acute effects of IL-1R endothelial signalling following ischaemic insult in SCD. As anakinra is an effective IL-1R antagonist in mice,[10,11] this drug may be useful to further explore the effects of more prolonged global IL-R antagonism on anaemic parameters in SCD mice. This pharmacological strategy would also mimic effects of treatments targeting the IL-1R in humans with SCD. Others have shown that IL-1β and platelet IL-1R regulate platelet activation, cytokine release, and transcript profile during inflammation in both mice and humans.[12] Thus, IL-1R expression on leucocytes and/or platelets may regulate release of cytokines such as transforming growth factor-β (TGF-β) that could promote erythrocyte generation of reactive oxygen species (ROS) leading to increased sickling.

To therefore test the potential effects of global IL-1R inhibition on anaemic parameters in SCD, we treated SCD mice with the IL-1R antagonist protein, anakinra. To determine the relevant circulating IL-1R cellular pool on erythrocyte sickling, *ex vivo* assays were performed and downstream mediators of IL-1R activity on sickling were explored.

Methods

Animals

Male C57BL/6J (wild-type, *Wt*, stock #000664), interleukin-1 receptor-1 null (*IL-1R$^{-/-}$*, stock #003245), and homozygous SCD mice (SCD, stock #013071, Townes model) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animal use protocols complied with the Principle of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Institutional Committee on Use and Care of Animals.

Anakinra treatment

Anakinra (10 mg/kg; Swedish Orphan Biovitrum AB, Sweden) or PBS (pH 7·4, Gibco, Carlsbad, CA, USA) was injected daily (intraperitoneal, i.p.) into SCD mice for 2 or 4 weeks.

Full blood counts

Blood samples were withdrawn from the retro-orbital venous plexus into ethylenediamine tetra-acetic acid (EDTA)-lined polythene tubes and were analysed using a Hemavet 950 (Drew Scientific, Inc., Miami Lakes, FL, USA). An aliquot of whole blood was mixed with new methylene blue (Ricca Chemical Company, Arlington, TX, USA) for 20 min to stain reticulocytes, reported as the percentage of stained to unstained erythrocytes.

Plasma haemolysis markers

Plasma was collected after mice were anesthetised with sodium pentobarbital (67 mg/kg, i.p.), from blood drawn from the inferior vena cava directly into 3·2% sodium citrate (9:1 blood/citrate ratio). Samples were centrifuged at 7700 g for 20 min and plasma was transferred to a new tube. Plasma haemoglobin (Hb) was detected using the Harboe direct spectrophotometric method with Allen correction[13] using the following equation: Hb (g/l) = $(167\cdot2 \times A_{415} - 83\cdot6 \times A_{380} - 83\cdot6 \times A_{450}) \times 1/1000 \times 1/\text{dilution}$ in distilled H_2O. Concentrations of plasma aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were determined by the University of Michigan Hospital laboratory.

Ex vivo *sickling assays*

Anakinra- and vehicle-treated SCD mice were anesthetised using isoflurane, then blood was drawn from the retro-orbital venous plexus into 3·2% sodium citrate (9:1 blood/citrate ratio). A 5 μl aliquot of blood was then placed on a slide with 5 μl of sodium metabisulphite [$Na_2S_2O_5$, Sigma, St. Louis, MO, USA; 2% w/v in phosphate-buffered saline (PBS)], a cover slip applied, then three images/slide were captured at ×20 after a 2-h incubation at room temperature. Sodium metabisulphite is a reducing agent that scavenges oxygen, promoting deoxygenation in sickle cells.[14,15] For *ex vivo* treatment, blood collected from untreated SCD mice was incubated with anakinra (149·25 ng/ml) or PBS for 10 min, before the addition of PBS or IL-1β (0·01 or 0·1 nM) for 2 h at room temperature. In a similar manner, 10-ul whole blood aliquots from *IL-1R$^{-/-}$* or *Wt* mice were mixed with 5 ul of washed SCD erythrocytes and then incubated with PBS or IL-1β (0·01 or 0·1 nM) for 2 h at room temperature. At the end of each respective 2-h incubation, a 5 μl aliquot of blood was then placed on a slide with 5 μl of 2% sodium metabisulphite, a cover slip applied, then three images/slide were captured at ×20 using a Nikon SE upright microscope and a Nikon DS-Fi3 camera (Nikon, Tokyo, Japan). The percentage of sickled erythrocytes/image was quantified.

For fractionation of cell types, 1 ml of Histopaque-1077 (Sigma) was aliquoted for each mouse into a 5-ml round bottom tube. Whole blood from SCD, *Wt* or *IL-1R$^{-/-}$* mice (four/group) was layered on top of the Histopaque-1077, then centrifuged at 400 *g* for 30 min at room temperature with no brake applied. The top layer of platelet-rich plasma and the ring of mononuclear cells was moved to a new 5-ml round bottom tube, then centrifuged again at 120 *g* for 10 min at room temperature with no break applied to separate the mononuclear cells (pellet) from platelets (in supernatant). After transfer of the supernatant to a new tube, platelets were washed in PBS, re-centrifuged at 7700 g for 10 min to pellet the platelets, then resuspended in PBS prior to treatment with PBS or IL-1β for 2 h at room temperature.

In some experiments, the mononuclear cells and the bottom layer containing polymorphonuclear cells was collected from the Histopaque-1077 gradient, washed, and resuspended in PBS prior to treatment. In all experiments utilising washed SCD erythrocytes, blood was collected from SCD mice retro-orbitally under isoflurane anaesthesia into 3·2% sodium citrate (9:1 blood/citrate ratio). Citrated blood was then centrifuged at 110 g for 10 min, washed twice in PBS, then re-suspended in PBS at 50% haematocrit (Hct) prior to treatment. Sickling percentages were determined in the same manner as in the whole blood sickling assays, described above.

For kinetics experiments, platelets were incubated with or without 0·1 nM of IL-1β for 30 min or 2 h before the platelet supernatant was applied to erythrocytes for 2 h. In later experiments, PBS- or IL-1β- treated (30 min, 0·1 nM) platelet supernatants were incubated with 50 μM of either anti-TGF-β 1D11 antibody (#16-9243-85, Invitrogen, Carlsbad, CA, USA) or control immunoglobulin G (IgG) antibody (#16-4714-85, Invitrogen) then added to washed SCD erythrocytes for 2 h. Recombinant mouse TGF-β1 (#7666-MB, 0·025 ng/ml, R&D Systems, Minneapolis, MN, USA), apocynin (#4663, 1 mM, Tocris, Bristol, Avon, UK), superoxide dismutase (SOD; Sigma #S7571-30 KU, 500 u/ml), 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL; Sigma #176141, 335 μM), or the pan-PKC inhibitor Go6983 (#2285, 1 μM, Tocris) was added to washed SCD erythrocytes immediately before the addition of PBS- or IL-1β- treated (30 min, 0·1 nM) platelet supernatants. Washed SCD erythrocytes were incubated with the treatments and supernatants for 2 h at room temperature. Sickling percentages were determined in the same manner as in the whole blood sickling assays, described above.

Calcium assay

Upon completion of sickling assay, erythrocytes were centrifuged at 110 g for 10 min. The supernatant was removed and the pelleted erythrocytes were resuspended in 150 μl radioimmunoprecipitation assay (RIPA) buffer and stored at −80°C until use. A calcium assay kit (Sigma #MAK022) was used according to manufacturer's directions on 50 μl of each erythrocyte lysate to measure calcium content.

Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde (MDA) content of undiluted plasma from the anakinra-treated mice was determined using a TBARS [trichloroacetic acid (TCA) Method] Assay kit from Cayman chemicals (Ann Arbor, MI, USA; #700870), following the manufacturer's instructions.

Dihydroethidium (DHE) assay

Superoxide anion production from erythrocytes was determined using DHE (Invitrogen, #D11347), following the manufacturer's instructions. Briefly, erythrocytes were isolated and treated with platelet supernatant or ROS scavengers as described above. At the end of the incubation, DHE at the concentration of 10 μM was incubated for 30 min with the erythrocytes while protected from light. Cells were transferred to a black, clear-bottomed 384-well plate and optimised detection of the superoxide specific product 2-OH-ethidium (Ex/Em: 405/570) over the non-specific product ethidium was obtained, as previously described.[16]

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity assay

Erythrocytes were treated with platelet supernatant, as described above. Erythrocytes were centrifuged at 110 g for 10 min, the supernatant was removed, and cell pellets were re-suspended in lysis buffer, following the protocol to assess NOX activity as in Griendling *et al.*[17] The cell suspension was then dounced 50 times on ice. The homogenate protein content was measured by Pierce BCA protein assay kit (#23225, Thermo Fisher Scientific Inc., Waltham, MA, USA). NOX activity was measured by a luminescence assay, as in Griendling *et al.*,[17] with the modification of using 10 μl of homogenate and 90 μl of reaction buffer rather than 100 μl and 900 μl as in Griendling *et al.*[17] Luminescence was monitored on Spectra Max iD5 (Molecular Devices, San Jose, CA, USA). The among of superoxide produced was calculated by comparison with a standard curve generated using xanthine and xanthine oxidase as described by.[18]

TGF-β1 enzyme-linked immunosorbent assay (ELISA)

Upon completion of sickling assay, erythrocytes were centrifuged at 110 g for 10 min. Supernatants were removed and the pelleted erythrocytes were stored at −80°C until use. Both preparation of lysates and ELISA process was performed according to the manufacturer's instructions (Invitrogen, #BMS608-4).

Immunohistochemistry

Formalin-fixed spleens were embedded in paraffin and cut in 5-μm sections before staining using anti-TGF-β 1D11 antibody (Invitrogen, #16-9243-85, 1:100). A positive stained area was detected using a biotin-conjugated secondary antibody, images were acquired on a Nikon Microphot-SA (#15941, Nikon Instruments Inc., New York, NY, USA) with a MicroPublisher 3.3RTB camera, (#Q25984, Teledyne Qimaging, Surrey, BC, Canada) and analysed by Image J software (10 fields of view/mouse, ×20). A positive staining area was expressed as a percentage of the total area.

Statistical analysis

Data are represented as mean ± standard deviation (SD). Statistical analysis was carried out using GraphPad Prism

(GraphPad Software Inc., San Diego, CA, USA). Significance of *in vivo* experiments, Western blot densitometry, and calcium content were determined with a paired Student's *t*-test. For analysis of multiple groups, significance was determined by an independent two-way analysis of variance (ANOVA) or a one-way ANOVA, followed by a *post hoc* analysis with Turkey's and Sidak's multiple comparisons tests. Probability values of $P < 0.05$ were considered statistically significant.

Results

Effect of anakinra on markers of haemolysis and anaemia in SCD mice

The SCD mice were given daily i.p. injections of anakinra or vehicle for 2 weeks, then blood cell counts and haemolysis markers were assessed. There were no significant differences in circulating erythrocyte, leucocyte, or platelet counts. However, free Hb, AST activity, and LDH activity were found to be decreased in the plasma of mice treated with anakinra for 2 weeks (Table I).

After 4 weeks of treatment with anakinra, erythrocyte count, Hb and Hct were all increased compared to vehicle-treated SCD mice (Table II). Additionally, reticulocyte percentages were decreased in anakinra-treated mice (Table II), suggesting reduced haemolysis. Circulating concentrations of

Table I. Markers of haemolysis.

	PBS	Anakinra	*P*
Mean (SD)			
Plasma Hb, ug/l	72·76 (43·26)	0·70 (13·44)	*0·007***
LDH, iu/l	935·4 (216·13)	524·33 (64·28)	*0·03**
AST, iu/l	317·4 (80·12)	135·66 (9·24)	*0·02**

The mean (SD) for each marker of haemolysis is indicated in the table (four/group) for sickle cell disease mice after 2 weeks of anakinra treatment (10 mg/kg daily, intraperitoneal). AST, aspartate aminotransferase; Hb, haemoglobin; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline.
*$P < 0.05$; **$P < 0.01$.

Table II. Effect of anakinra treatment on anaemia.

	PBS	Anakinra	*P*
Mean (SD)			
Hb, g/l	77.2 (1.3)	98 (8.6)	*0·030**
Hct, %	32·06 (0·93)	46·25 (4·03)	*0·040**
RBC count, $\times 10^{12}$/l	6·77 (0·18)	9·01 (0·78)	*0·048**
Retic., %	32·45 (2·74)	25·31 (1·79)	*0·018**

The mean (SD) values are given in the table (four/group) for sickle cell disease mice after 4 weeks of anakinra treatment (10 mg/kg daily, intraperitoneal). Hb, haemoglobin; Hct, haematocrit; PBS, phosphate-buffered saline; RBC, red blood cell; Retic., reticulocyte.
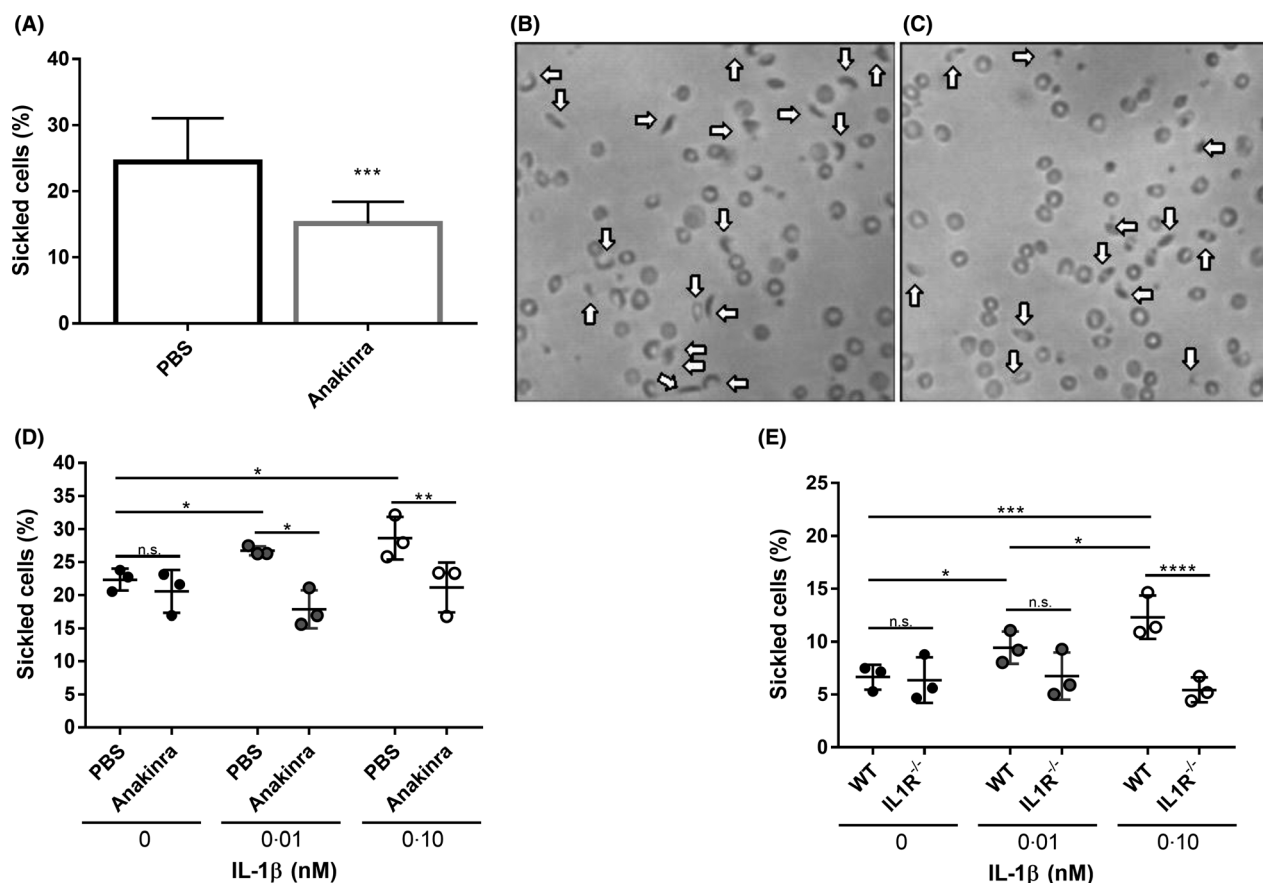*$P < 0.05$.

platelets [mean (SD) 1233 (74·61) vs. 1196 (152·20) $\times 10^9$/l, $P = 0.58$] were not different between anakinra and vehicle-treated mice.

IL-1β promotes sickling in whole blood ex vivo

To test whether the improved anaemia observed in anakinra-treated mice was associated with reduced erythrocyte sickling, a sickling assay was performed with whole blood from treated SCD mice using sodium metabisulphite, a reducing agent which promotes deoxygenation in sickle erythrocytes by scavenging oxygen.[14,15] The percentage of sickled erythrocytes was decreased in blood from anakinra-treated mice compared to controls (Fig 1A–C). Next, whole blood from untreated SCD mice was incubated for 10 min with PBS or anakinra, before the addition of IL-1β (0, 0·01, or 0·10 nM IL-1β, final concentration). A dose-related increase in sickling was observed in samples pre-treated with PBS, while this was blocked in samples pre-treated with anakinra (Fig 1D). To further verify the specificity of the sickling response to IL-1β, whole blood was collected from *Wt* or *IL-1R*$^{-/-}$ mice, to which washed SCD erythrocytes were added. Basal sickling rates were not different between SCD erythrocytes incubated with *Wt* or *IL-1R*$^{-/-}$ whole blood (Fig 1E). After the addition of IL-1β a dose-response increase in sickling was observed in *Wt* blood samples, that was not observed in *IL-1R*$^{-/-}$ blood samples (Fig 1E).

IL-1β promotes sickling indirectly through action on platelets

To determine if IL-1β could directly promote *ex vivo* sickling, washed erythrocytes were prepared from SCD mice and then pre-treated with PBS or anakinra for 10 min, before the addition of PBS or IL-1β. In contrast to the previous whole blood experiments, the sickling rates of washed erythrocytes were unresponsive to IL-1β or anakinra (Fig 2). This suggests that IL-1β indirectly promotes sickling by acting on a separate cell population. Therefore, to determine the relevant cellular pool for IL-1β effects on *ex vivo* sickling, cell fractions were prepared from SCD blood using Histopaque-1077, and then treated with PBS or IL-1β. After treatment, cells were centrifuged, and the supernatants were applied to washed SCD erythrocytes. As shown in Fig 2, erythrocytes incubated without supernatant or with supernatant from polymorphonuclear or mononuclear cells did not respond to IL-1β. However, supernatants from platelet cell fraction showed a dose–response increase in sickling when applied to washed SCD erythrocytes (Fig 2).

The platelets of patients with sickle cell are functionally hyperactive.[19,20] To further delineate if the platelet-mediated ability to induce sickling in response to IL-1β was unique to SCD platelets, the same experiment was performed with *Wt* cell fractions. Similar to SCD cell fractions, supernatants prepared from IL-1β-treated mononuclear cells did not produce

Fig 1. Whole blood sickling assays with Anakinra. (A) Mice were given daily intraperitoneal injections with vehicle [phosphate-buffered saline (PBS)] or anakinra for 4 weeks. The percentage of sickled cells were quantified (three fields of view/mouse, four mice/group) and the mean ± standard deviation (SD) is shown; significance determined by an unpaired Student's *t*-test. Representative images of blood cells from PBS-treated mice (B) and anakinra-treated mice (C) are shown. Arrows indicate sickled cells. (D) Whole blood from untreated sickle cell disease (SCD) mice was drawn and treated *ex vivo* with ± anakinra ± interleukin 1 beta (IL-1β) before the percentage of sickled cells was assessed. Mean ± SD is shown (*n* = 4). Significance was determined by an independent two-way analysis of variance (ANOVA), followed by a *post hoc* analysis with Turkey's and Sidak's multiple comparisons tests. (E) Washed SCD red blood cells were incubated with whole blood from either wild-type (WT) or interleukin-1 receptor (IL-1R)$^{-/-}$ mice at a ratio of 1:2 in the presence of varied concentrations of IL-1β before assessment of sickling. The mean of the percentage of sickled cells ± SD is shown. Significance was determined by an independent two-way ANOVA, followed by a *post hoc* analysis with Turkey's and Sidak's multiple comparisons tests. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

increased sickling when incubated with SCD erythrocytes (Table III). However, supernatants from IL-1β-treated platelets did promote the dose–response increase in sickling, which was not observed in IL-1β-treated platelet supernatants from *IL-1R*$^{-/-}$ mice (Table III). Therefore, the release of the sickling factor from platelets in response to IL-1β is dependent on IL-1R presence and is not a unique response of SCD platelets.

Kinetics of IL-1β-induced platelet effect on erythrocyte sickling

To gain insight into the kinetics of the response of platelets to IL-1β, platelets were incubated with IL-1β for 30 min or 2 h before the platelet supernatant was applied to erythrocytes for 2 h. The supernatant from platelets incubated with IL-1β for 30 min was sufficient to generate

a similar sickling response as a 2 h IL-1β incubation (Table IV).

Superoxide generation in response to IL-1β *in vitro* and in vivo

Reactive oxygen species have been shown to promote erythrocyte membrane rigidity,[21] mechanical instability,[22] and to oxidise Hb.[22,23] Treatment of sickle red blood cells (RBCs) with antioxidants can prevent irreversible sickling *in vitro*.[24] We therefore hypothesised that the increase in sickling may occur through increased presence of ROS post-supernatant treatment. Superoxide anion presence was assessed via DHE fluorescence and found to be increased in erythrocytes after incubation with the IL-1β-treated platelet supernatant relative to PBS-treated samples (Fig 3). Apocynin, an inhibitor of superoxide generation by NOX, and Tempol, a SOD mimetic,
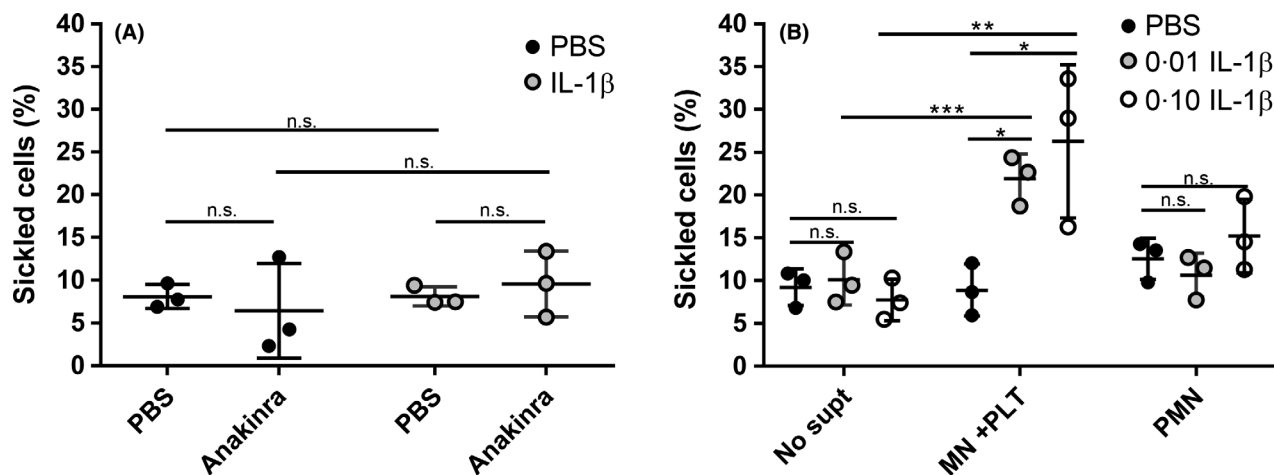
Fig 2. Interleukin 1 beta (IL-1β) indirectly promotes *in vitro* sickling. (A) Washed sickle cell disease (SCD) red blood cells (RBCs) were incubated with ± anakinra (149·25 nM) for 10 min before incubation ± IL-1β (0, 0·01, 0·1 nM) for 2 h. Then, the percentage of sickled cells was assessed. Mean ± standard deviation is shown ($n = 4$) and is not significantly different. (B) Cell fractions were collected, incubated ± IL-1β (0, 0·01, 0·1 nM) for 2 h, and then treated supernatants were applied to washed SCD RBCs for another 2 h before the percentage of sickled cells was assessed. Significance was determined by an independent two-way analysis of variance (ANOVA), followed by a *post hoc* analysis with Turkey's and Sidak's multiple comparisons tests. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$, ***; $P < 0.005$; ****, $P < 0.0001$.

Table III. *In vitro* sickling assays with washed red blood cells and fractionated cell supernatants.

	Treatment with IL-1β (nM)			*P*	
	0	0·01	0·1	0–01	0–0·1
Mean (SD)					
No supt.	9·23 (3·75)	10·12 (2·97)	7·74 (2·84)	0·6423	0·3780
Wt PMN supt.	12·54 (2·40)	10·64 (2·57)	15·22 (4·29)	0·3171	0·3585
Wt MN supt.	12·22 (2·67)	14·41 (2·62)	14·09 (2·05)	0·3420	0·4170
Wt PLT supt.	10·17 (1·16)	13·72 (4·64)	22·85 (1·26)	0·0206*	0·0002***
IL-1R$^{-/-}$ MN supt.	9·20 (2·52)	12·44 (2·46)	11·11 (0·68)	0·4930	0·2660
IL-1R$^{-/-}$ PLT supt.	11·79 (0·89)	11·39 (2·65)	12·64 (4·64)	0·8380	0·7090

Whole blood was fractionated into different cell populations. The isolated cell populations were treated for 2 h with indicated concentrations of mouse recombinant interleukin 1 beta (IL-1β) before centrifugation to remove cells from supernatants. The supernatants were applied to washed sickle cell disease red blood cells (RBCs) and incubated for 2 h, after which time sickling was assessed. The mean (SD) of the percentage of sickled RBCs is indicated in the table (three/group). Significance was determined by a one-way analysis of variance (ANOVA), followed by a *post hoc* analysis with Turkey's multiple comparisons tests. IL-1R, interleukin-1 receptor; MN, mononuclear cells; PLT, platelet; PMN, polymorphonuclear cells; supt., supernatant; *Wt,* wild-type.
*$P < 0.05$; ***$P < 0.005$.

were used as negative controls to verify specificity of superoxide assessment. Inhibition of IL-1R1 with anakinra *in vivo* also decreased plasma ROS as assessed by plasma MDA, a major lipid oxidation product. This decrease in plasma MDA was observed in anakinra-treated mice compared to vehicle-treated mice following both 2 and 4 weeks of treatment (Fig 3).

Platelet supernatant-mediated sickling is abrogated by treatment with anti-TGF-β antibody

TGF-β has been reported to increase ROS in sickle erythrocytes via activation of NOX, a primary source of superoxide

ions in sickle erythrocytes.[25] TGF-β is released from stimulated platelets;[26] however, the ability of IL-1β to promote the release of platelet TGF-β had not yet been assessed. We hypothesised that in response to IL-1β, platelet-derived TGF-β would promote erythrocyte NOX activity, which would then increase superoxide production leading to erythrocyte sickling. To first address if alterations to this proposed pathway affected IL-1β-mediated sickling, washed erythrocytes were incubated with platelet supernatants that contained anti-TGF-β antibody 1D11 or control IgG. The IL-1β-mediated increase in sickling was abrogated by the presence of anti-TGF-β antibody (Fig 4A) but not by an anti-IgG

Table IV. *In vitro* sickling assays for 30 min or 2 h incubation with platelet supernatants.

	PBS	IL-1β (0·1 nM)	P
Mean (SD) % sickled RBCs at:			
30 min	10·73 (0·82)	17·19 (1·04)	0·001**
2 h	11·15 (1·08)	18·38 (1·75)	0·009**

Isolated platelets were treated for 30 min or 2 h with indicated concentrations of mouse recombinant interleukin 1 beta (IL-1β) before centrifugation to remove cells from supernatants. The supernatants were applied to washed sickle cell disease red blood cells (RBCs) and incubated for 2 h, after which time sickling was assessed. The mean (SD) of the percentage of sickled RBCs are given in the table (three/group). Significance was determined by a one-way analysis of variance (ANOVA), followed by a *post hoc* analysis with Turkey's multiple comparisons tests. PBS, phosphate-buffered saline.
**$P \leq 0.01$.

control antibody (Fig 4A). The inclusion of recombinant TGF-β increased sickling relative to PBS-treated platelet supernatants, to a similar percentage as IL-1β-treated supernatants (Fig 4A). This TGF-β-mediated increase in sickling could be inhibited by co-culture with anti-TGF-β antibody. Additionally, apocynin blocked the sickling effect of IL-1β. Combined treatment of erythrocytes with anti-TGF-β antibody and apocynin did not further decrease sickling relative to each treatment alone (Fig 4A). IL-1β-treated platelet supernatant induced an increase in NOX activity in erythrocytes after 2 h of treatment relative to controls (Fig 4B). To further verify the reliance of IL-1β-mediated sickling on superoxide generation, SOD or the ROS scavenger and SOD mimetic, Tempol, was applied to erythrocytes concurrently with IL-1β- or PBS-treated platelet supernatants. In the presence of Tempol or SOD, IL-1β-mediated sickling was

inhibited (Fig 4C). Finally, the increase in sickling observed with recombinant TGF-β incubation was abrogated by apocynin co-treatment (Fig 4C).

To verify the transfer of TGF-β to erythrocytes, the relative concentration of TGF-β present in washed erythrocytes after incubation with IL-1β, treated platelet supernatants were assessed via ELISA. The TGF-β concentration was increased in erythrocyte lysates that had received IL-1β-treated platelet supernatant relative to controls (Fig 5A). However, in the plasma of mice treated with anakinra for 2 weeks, the plasma concentration of TGF-β was not decreased relative to vehicle treated mice as assessed by ELISA [mean (SD) 182·82 (18·98) vs. 183·85 (22·18) pg/ml]. TGF-β has been shown to be expressed at higher levels in the spleen compared to other organs in adult mice, likely due to platelet release.[27] Spleens from vehicle and anakinra-treated mice were immunostained for TGF-β and reduced TGF-β staining was observed in spleens from anakinra-treated mice (Fig 5B). Finally, NOX activation by plasma factors has been shown to occur through protein kinase C (PKC) activation in a pathway requiring increase intracellular calcium.[25] PKC inhibition by Go6983 (1 μM, as previously[28]) was observed to abrogate the sickling response of IL-1β-treated platelet supernatants (Fig 5C). In support of this mechanism, intracellular calcium was also observed to increased significantly in erythrocyte lysates after incubation with IL-1β-treated platelet supernatants (Fig 5D).

Discussion

Haemolysis promotes inflammasome activation with increased IL-1β production.[29] Subsequent activation of IL-1β signalling pathways may contribute to organ damage and perpetuate haemolysis in chronic haemolytic syndromes such as SCD.[29,30] IL-1β signals through the IL-1R to trigger
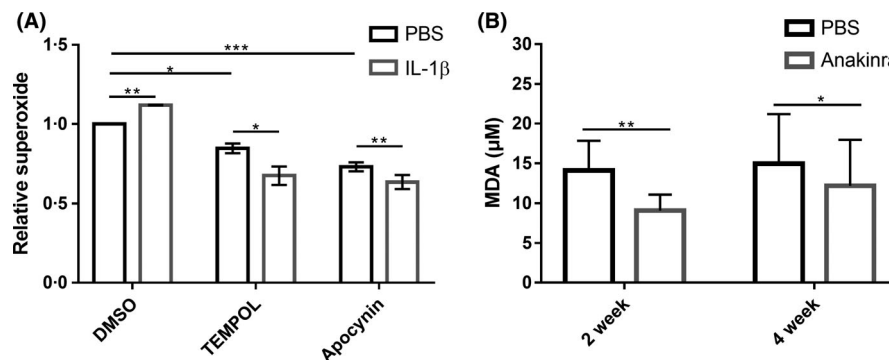

Fig 3. Alterations to reactive oxygen species in response to interleukin 1 beta (IL-1β) or anakinra (A) Dihydroethidium (DHE) at the concentration of 10 μM was incubated for 30 min with the erythrocytes while protected from light. Cells were transferred to a black, clear-bottomed 384-well plate and the optimised detection of the superoxide specific product 2-OH-ethidium (Ex/Em: 405/570) over the unspecific product ethidium was obtained. Mean ± standard deviation (SD) is shown (three/group). Significance was determined by an independent two-way analysis of variance (ANOVA), followed by a *post hoc* analysis with Turkey's and Sidak's multiple comparisons tests. (B) Mean plasma malondialdehyde (MDA) concentrations ± SD are plotted from mice receiving anakinra for 2 weeks or 4 weeks, $n = 4$ each group. Significance was determined with Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. DMSO, dimethyl sulphoxide.
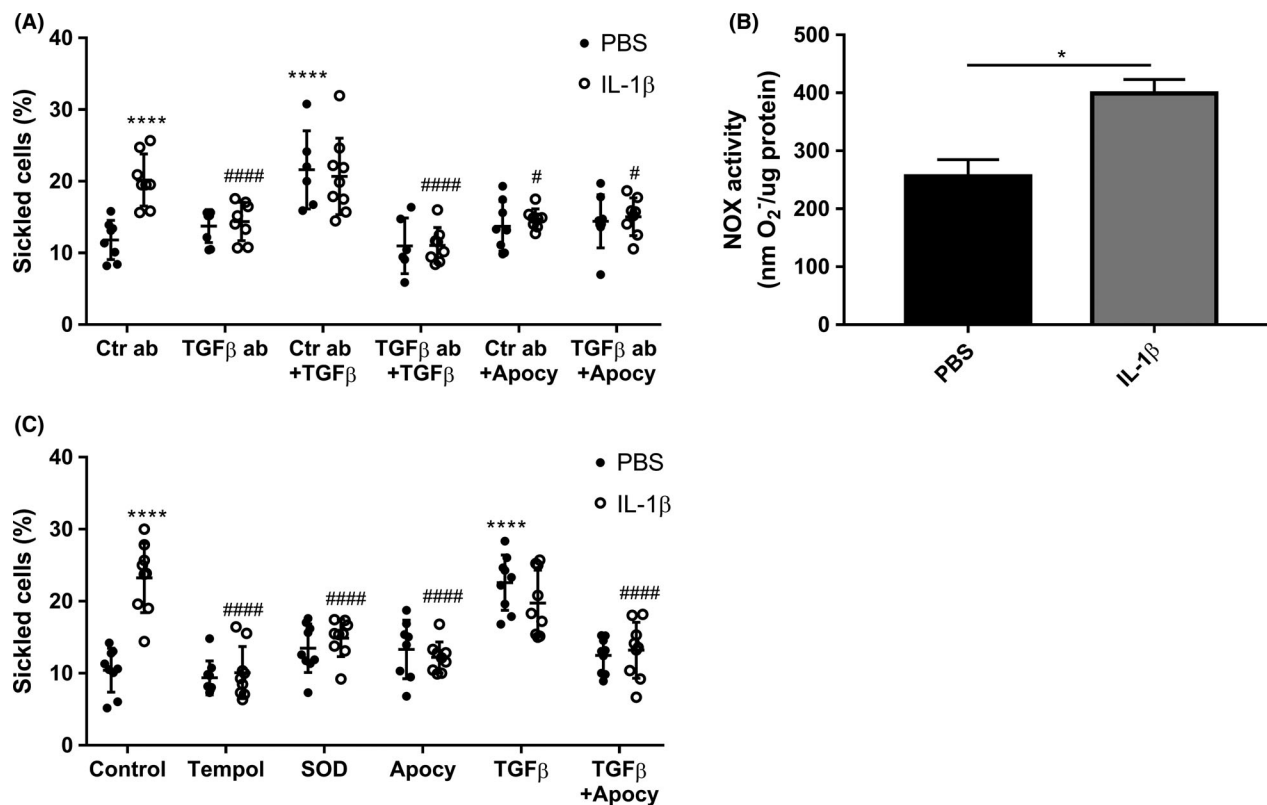
Fig 4. Perturbation of interleukin 1 beta (IL-1β)-mediated sickling with anti-transforming growth factor-β (TGF-β) antibody, nicotinamide adenine dinucleotide phosphate oxidase (NOX) inhibition, or reactive oxygen species scavengers. (A) Washed sickle cell disease (SCD) red blood cells (RBCs) ± inhibitory antibodies, recombinant TGF-β, or apocynin were incubated for 2 h with supernatants from platelets treated for 30 min ± IL-1β. The mean ± standard deviation (SD) of the percentage of sickled cells is shown; each dot indicates the percentage of sickled cells for a specific field of view (two to three fields of view/mouse, three mice/group). ****, $P < 0.0001$ relative to phosphate-buffered saline (PBS)-treated platelet supernatant + control antibody. #, $P < 0.05$; ####, $P < 0.0001$ relative to IL1β-treated platelet supernatant + control antibody. (B) Washed SCD RBCs were incubated for 2 h with supernatants from platelets treated for 30 min ± IL-1β, then erythrocytes were lysed on ice and the lysates were tested for NOX activity. The mean ± SD of superoxide anion (nM) generated/μg protein is shown; three technical replicates/mouse, $n = 5$ mice). Significance was determined using Student's t-test, $P < 0.05$. (C) Washed SCD RBCs ± Tempol, superoxide dismutase (SOD), apocynin or recombinant transforming growth factor-β (TGF-β) were incubated for 2 h with supernatants from platelets treated for 30 min ± IL-1β. The mean ± SD of the percentage of sickled cells is shown; each dot indicates the percentage of sickled cells for a specific field of view (three fields of view/mouse, three mice/group). Significance was determined by an independent two-way analysis of variance (ANOVA), followed by a *post hoc* analysis with Turkey's and Sidak's multiple comparisons tests. ****, $P < 0.0001$ relative to PBS-treated platelet supernatant. ####, $P < 0.0001$ relative to IL-1β-treated platelet supernatant.

inflammatory responses, which are mediated by multiple cell types.[31–34] Some limited evidence for a causal role for IL-1β signalling in SCD pathology prior to this study include the ability of sickled erythrocytes to induce NLRP3 inflammasomes with increased IL-1β expression in peripheral blood mononuclear cells.[35]

Deficiency of IL-1R limited to non-haematopoietic cellular pools has shown to be beneficial in reducing haemolysis-related organ damage and stroke size in a murine model of SCD.[6] These beneficial effects are likely caused by reduced IL-1β-mediated induction of endothelial adhesion molecules. In this non-haematopoietic IL-1R deficiency state, no effect was observed on anaemic parameters.[6] To determine the effects of global IL-1R inhibition on anaemia in SCD, mice were treated for several weeks with anakinra in the present study. After 2 weeks of treatment, no difference in anaemic parameters

(Hb, Hct, RBC) were observed between mice treated with anakinra or vehicle control; however, circulating levels of serum AST, LDH, and cell-free haemoglobin were reduced in anakinra-treated mice, suggesting reduced haemolysis. After 4 weeks of treatment, anaemia was improved in anakinra-treated mice while markers of haemolysis remained reduced indicating global IL-1R antagonism might improve anaemia via reduced haemolysis in SCD. As non-haematopoietic genetic deficiency of IL-1R did not affect anaemia in SCD,[6] these findings suggest haematopoietic expression of IL-1R promotes haemolysis and anaemia in SCD.

Erythrocytes in blood extracted from SCD humans or mice undergo *ex vivo* sickling, which may be reflective of the *in vivo* sickling tendency.[36,37] Consistent with the reduced haemolysis observed *in vivo* following anakinra treatment, erythrocyte sickling in whole blood was reduced
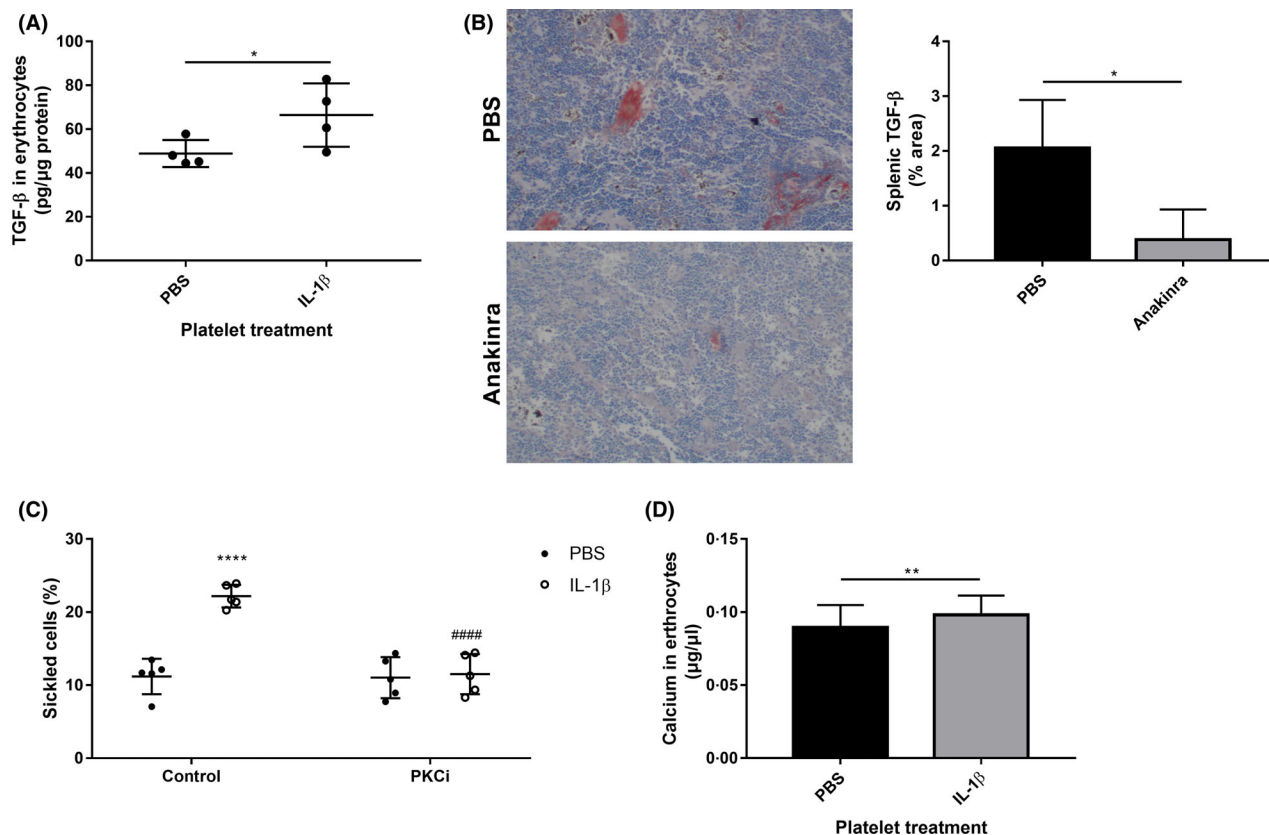
Fig 5. Transforming growth factor-β (TGF-β) content downstream of interleukin 1 beta (IL-1β) manipulation and involvement of protein kinase C (PKC) in the proposed mechanism. (A) Enzyme-linked immunosorbent assay of total TGF-β concentration ± standard deviation (SD) of erythrocyte lysates collected after a 2 h incubation with supernatants from platelets treated for 30 min ± IL-1β (four/group). Significance was determined by Student's *t*-test, *, $P < 0.05$. (B) Representative images of spleens immunostained for TGF-β after 2 weeks treatment with or without anakinra. Graph depicts percentage splenic area positive for TGF-β ± SD, four mice/group. Significance was determined by Student's *t*-test, *, $P < 0.05$. (C) Sickling assay including pan-PKC inhibition with G06983 (1 μM). Significance was determined by an independent two-way analysis of variance (ANOVA), followed by a *post hoc* analysis with Turkey's and Sidak's multiple comparisons tests. ****, $P < 0.0001$ relative to phosphate-buffered saline (PBS)-treated platelet supernatant. ####, $P < 0.0001$ relative to IL-1β-treated platelet supernatant. (D) Calcium concentrations of erythrocyte lysates after incubation with platelet supernatants ± IL-1β for 2 h (four/group). Significance was determined with Student's *t*-test, **, $P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com]

in anakinra-treated mice. IL-1R can be activated by binding either IL-1α or IL-1β.[38] While the addition of exogenous IL-1β to whole blood of SCD mice produced a dose-dependent increase in erythrocyte sickling that was blocked by pre-incubation with anakinra *in vitro*, we cannot rule out effects of IL-1α *in vivo*. Similarly, when washed SCD erythrocytes were co-incubated with whole blood obtained from either *IL-1R*^{+/+} or *IL-1R*^{−/−} mice, a dose-dependent effect of exogenous IL-1β was observed on sickling in the *IL-1R*^{+/+} whole blood group while no effect of IL-1β on sickling was observed in the *IL-1R*^{−/−} whole blood group. Taken together, these findings implicate a circulating IL-1R-expressing cell population capable of promoting erythrocyte sickling in response to IL-1β. No effect of IL-1β on sickling was observed when washed SCD erythrocytes were incubated with IL-1β alone, without other blood cell types present, suggesting IL-1β does not act directly on erythrocytes to promote sickling. To determine which circulating blood cell type

was responsible for the effects on sickling in this assay, supernatants were generated from polymorphonuclear cells, mononuclear cells, or platelets, with and without IL-1β stimulation. After incubation with IL-1β, only supernatants prepared from platelets promoted erythrocyte sickling. The platelet supernatant effect was absent when platelets from *IL-1R*^{−/−} mice were used, indicating the platelet IL-1R pool may be responsible for the anakinra effect on anaemia. As the effect occurred upon transfer of supernatants, this suggests cell contact between platelets and erythrocytes is not necessary for the effect, but rather that a factor is released from platelets that promotes SCD sickling *in vitro*. Figure 6 depicts the proposed mechanism for the observed IL-1β-mediated sickling.

Platelets express the IL-1R and signal in response to IL-1β.[12] The platelet IL-1R has been shown to regulate platelet activation and platelet transcript profile during inflammation in mice and humans,[12] and to contribute to vascular

thrombosis.[39] One of the actions of IL-1β on platelets is the stimulation of IL-1β synthesis through an autocrine signalling loop involving caspase-1.[12] Platelet caspase-1 activity has been shown to be increased upon incubation with SCD plasma[2] and treatment of SCD mice with a caspase-1 inhibitor reduced pulmonary vaso-occlusion[30] Potential effects of caspase-1 inhibition on SCD anaemia through the signalling pathways demonstrated here warrants further study. Recently, the ability of platelet inflammasomes to worsen SCD phenotypes through generation of platelet-derived extracellular vesicles has been described,[30] although the presence of inflammasomes in platelets is still contentious.[40] A potential role of extracellular vesicles towards the release or activation of TGF-β by platelets within 30 min of IL-1β stimulation was not addressed in the present study.

Platelet's release TGF-β and platelet-generated TGF-β contributes largely to TGF-β plasma levels.[41] A neutralising antibody to TGF-β attenuated the effect of IL-1β-stimulated platelet supernatant on erythrocyte sickling, supporting the hypothesis of platelet release of activated TGF-β. The TGF-β antibody used in the present study is capable of inhibiting all three TGF-β isoforms; however, TGF-β1 is most widely reported in platelets.[26,41,42] Upon incubation of recombinant mouse TGF-β1 with sickle erythrocytes, an increase in sickling was observed. Erythrocyte calcium was observed to increase after incubation with IL-1β-stimulated platelet supernatant, consistent with the previous report describing the mechanism of TGF-β-mediated stimulation of erythrocyte NOX through PKC activation and increased intracellular calcium.[25] Although the changes in intracellular calcium were modest (9·6% increase over control values), intracellular calcium concentrations are tightly controlled and even small changes in erythrocyte calcium can have pronounced effects.[43] Intracellular calcium increases in erythrocytes is associated with sickling[44] as well as with erythrocyte adhesion.[45] Erythrocytes contain several isoforms of PKC: PKCα, PKCζ, PKCι and PKCμ.[46] Further studies are necessary to determine which PKC is most relevant towards this phenotype. In the present study, erythrocyte-associated TGF-β was observed to increase after treatment with stimulated platelet supernatant; however, it is possible that platelets may respond to IL-1β with the release of other factors that may activate latent TGF-β and could contribute to this phenotype. While reduced TGF-β levels were not observed in the plasma of anakinra-treated mice, reduced TGF-β expression in response to anakinra was observed in the spleen, an organ known for its large platelet content.[47] Additional studies with genetically manipulated mouse models such as platelet-specific TGF-β null mutants will be necessary to confirm the in vivo significance of these findings.

While sickle haemoglobin auto-oxidation and Fenton chemistry reactions generate excess ROS in SCD erythrocytes,[48,49] another key source of ROS in these cells is NOX activity.[25] NOX activation appears to play a causal role in platelet supernatant-mediated erythrocyte sickling, as NOX activity was increased in erythrocytes in response to IL-1β-stimulated platelet supernatant, and the NOX inhibitor, apocynin, blocked the effect of IL-1β on platelet supernatant-mediated sickling. The ability of SOD or the cell permeable SOD mimetic, Tempol, to mitigate the sickling response also supports a role of superoxide on IL-1β-mediated sickling. As further evidence of this mechanism, erythrocytes incubated with supernatants harvested from IL-1β-stimulated platelets produced more superoxide as indicated by DHE fluorescence. Inhibition of IL-1R signalling with anakinra would also be expected to decrease ROS, and indeed, in mice treated with anakinra the lipid peroxidation product MDA[50] was found to be reduced relative to controls. While the net effect of IL-1β-stimulated platelet releasate was the generation of superoxide, this releasate is a complex milieu and there may be both pro- and anti-oxidant effects. Indeed, IL-1β in combination with Tempol or apocynin decreased ROS relative to cells treated with Tempol and apocynin alone. This finding may represent other less potent anti-oxidant factors present in stimulated platelet lysates.

Limitations of the present study include pharmacological antagonism of candidate cytokines/receptors, which may have off-target effects in vivo. Additionally, in vitro sickling assays
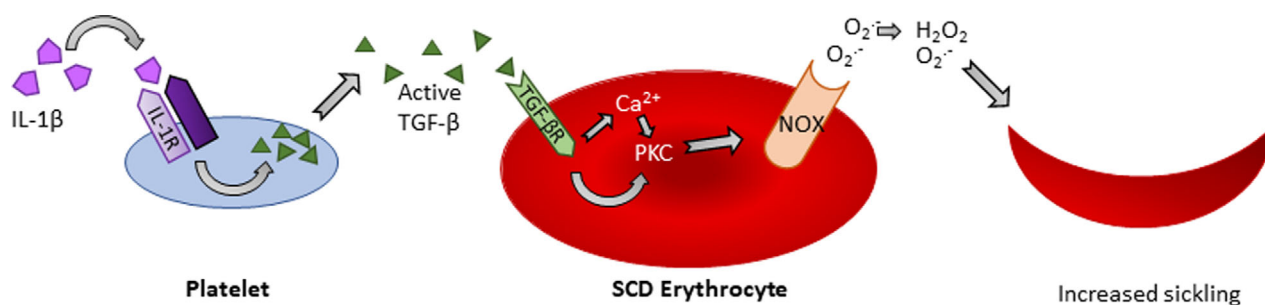

Fig 6. Proposed model for interleukin 1 beta (IL-1β)-mediated increase in sickling. In response to IL-1β stimulation, platelets release active transforming growth factor-β (TGF-β) among other possible factors. TGF-β released from IL-1β-treated platelets leads to activation of erythrocyte protein kinase C (PKC) which further activates erythrocyte nicotinamide adenine dinucleotide phosphate oxidase (NOX). The superoxide ($O_2^{·-}$) produced by activated NOX, and the hydrogen peroxide (H_2O_2) produced from $O_2^{·-}$, promote erythrocyte sickling. The observed increase in calcium may promote PKC activity and independently promote erythrocyte sickling. [Colour figure can be viewed at wileyonlinelibrary.com]

were used to explore cell types and downstream pathways related to the IL-1R and TGF-β. Genetic deletion models, global and tissue-specific, will be helpful in confirming the effects of the IL-1R and TGF-β on relevant end-points in murine models of SCD. Additionally, while the excitation and emission wavelengths used to detect superoxide were optimised to detect the superoxide-generated product of DHE,[16] the input of other ROS cannot be completely ruled out with this method. Similarly, the specific input from other ROS may account for an unknown amount of the MDA concentration observed in anakinra-treated mice. Leucocyte-generated ROS may also be inhibited after anakinra treatment and could explain the *in vivo* results. Finally, platelets may release other factors that contribute to ROS generation,[51,52] which could also promote erythrocyte sickling in SCD through membrane oxidative injury.[5]

In conclusion, pharmacological antagonism of the IL-1R in SCD mice improves anaemia. This effect may be related to the inhibition of IL-1β stimulation of platelets with subsequent release of TGF-β and erythrocyte ROS generation. Additional studies with genetically manipulated mouse models, e.g. platelet-specific TGF-β-null mutants, will be necessary to confirm the *in vivo* significance of these findings. The clinical implications are that short-term treatment strategies targeting IL-1R, TGF-β or downstream effector molecules may be beneficial in managing complications of SCD.

Acknowledgements

Conflicts of interest

No conflicts of interest to disclose.

Author contributions

Jessica Venugopal planned and performed experiments, collected and analysed the data, interpreted the data, and co-wrote the manuscript. Jintao Wang and Chiao Guo performed experiments and collected data. Daniel T. Eitzman conceived and co-ordinated the study, interpreted the data and co-wrote the manuscript. All authors discussed the results and commented on the manuscript.

Data availability statement

For original data, please contact deitzman@umich.edu.

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