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5 Article type : Ordinary Papers

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8 Interleukin-1 receptor antagonism leads to improved anemia in a murine model of 9 sickle cell disease and is associated with reduced ex vivo platelet-mediated 10 erythrocyte sickling

11 *Running title: Interleukin-1 receptor in SCD anemia*

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/BJH.17941

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3 **Data sharing:** For original data, please contact deitzman@umich.edu.

4 **Abstract word count:** 202

5 **Total word count:** 4341

6 **Figures and Tables:** 9 (6 figures and 4 tables)

7 **Supplemental files:** 0

8 **Acknowledgements:** This work was supported by the National Institutes of Health
9 (T32-HL007853 to J.V.) and a VA Merit Award (BX002776 to D.T.E.).
10

11 **Disclosures:** No conflicts of interest to disclose.

12 **Contributions:** JV planned and performed experiments, collected and analyzed the
13 data, interpreted the data, and co-wrote the manuscript. JW and CG performed
14 experiments and collected data. DTE conceived and coordinated the study, interpreted
15 the data and co-wrote the manuscript. All authors discussed the results and commented
16 on the manuscript.

17 **Abstract**

18 Sickle cell disease (SCD) is associated with hemolytic anemia and secondary activation
19 of leukocytes and platelets, which in turn may further exacerbate hemolysis. Since
20 cytokine signaling pathways may participate in this cycle, the current study investigated
21 whether pharmacologic blockade of the interleukin-1 receptor (IL-1R) would mitigate
22 anemia in a murine model of SCD. Within 2 weeks of treatment, reduced markers of
23 hemolysis were observed in anakinra-treated mice compared to vehicle-treated mice.
24 After 4 weeks of anakinra treatment, mice showed increased numbers of erythrocytes,
25 hemoglobin, and hematocrit, along with reduced reticulocytes. Blood from anakinra-
26 treated mice was less susceptible to *ex vivo* erythrocyte sickling and was resistant to
27 exogenous IL-1β-mediated sickling. Supernatant generated from IL-1β-treated platelets

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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) neutralizing antibody, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibition, and superoxide scavengers, but replicated by recombinant TGF-β. In conclusion, pharmacologic IL-1R antagonism leads to improved anemia 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.

Introduction

Hemolytic anemia 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 anemia (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 SCD subjects (2-4). IL-1β is a particularly important mediator of hemolysis-related inflammation which may promote vaso-occlusion (1, 5). While these previous studies suggest IL-1β signaling 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-hematopoietic cellular IL-1R pools provide protection against organ damage and stroke (6). These effects appear to be mediated via reduced endothelial adhesive properties, limiting leukocyte extravasation at sites of injury in SCD mice. However, these experiments did not assess the effects of IL-1R signaling on hematopoietic cells due to the nature of the chimeric mice generated by bone marrow transplantation. Protective effects of IL-1R deficiency involving the hematopoietic cellular pool may also be relevant in SCD. The therapeutic targeting of IL-1β has proven beneficial in several inflammatory diseases (7-9). Pharmacologic inhibition of IL-1R with

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1 the IL-1R1 antagonist protein, anakinra, was shown to prevent the increased stroke size
2 in SCD mice when given as a single dose shortly after stroke induction (6), supporting
3 acute effects of IL-1R endothelial signaling following ischemic insult in SCD. Since
4 anakinra is an effective IL-1R antagonist in mice (10, 11), this drug may be useful to
5 further explore the effects of more prolonged global IL-R antagonism on anemic
6 parameters in SCD mice. This pharmacologic strategy would also mimic effects of
7 treatments targeting the IL-1R in humans with SCD. Others have shown that IL-1β and
8 platelet IL-1R regulate platelet activation, cytokine release, and transcript profile during
9 inflammation in both mice and humans (12). Thus, IL-1R expression on leukocytes
10 and/or platelets may regulate release of cytokines such as TGF-β that could promote
11 erythrocyte generation of reactive oxygen species (ROS) leading to increased sickling.

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

16

17

18 **Methods**

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

25 **Anakinra treatment** – Anakinra (10 mg/kg) (Swedish Orphan Biovitrum AB, Sweden) or
26 PBS (Gibco, pH 7.4) was injected daily (intraperitoneal, i.p.) into SCD mice for 2 or 4
27 weeks.

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Complete blood counts – Blood samples were withdrawn from the retro-orbital venous plexus into EDTA-lined polythene tubes and were analyzed using a Hemavet 950 (Drew Scientific, Inc). An aliquot of whole blood was mixed with new methylene blue (Ricca Chemical Company) for 20 minutes to stain reticulocytes, reported as the percentage of stained to unstained erythrocytes.

Plasma hemolysis markers – Plasma was collected after mice were anesthetized 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 8500 rpm for 20 min and plasma was transferred to a new tube. Plasma hemoglobin was detected using the Harboe direct spectrophotometric method with Allen correction (13) using the following equation: Hb (g/L) = (167.2 x A_{415} - 83.6 x A_{380} - 83.6 x A_{450}) x 1/1000 x 1/dilution in distilled H_20. 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 anesthetized using isofluorane, then blood was drawn from the retro-orbital venous plexus into 3.2% sodium citrate (9:1 blood/citrate ratio). A 5 uL aliquot of blood was then placed on a slide with 5 uL sodium metabasulfite ($Na_2S_2O_5$, Sigma, St. Louis, MO; 2% w/v in PBS), a cover slip applied, then 3 images per slide were captured at 20x after a 2 hour incubation at room temperature. Sodium metabasulfite is a reducing agent which 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 minutes, before the addition of PBS or IL-1β (0.01 or 0.1 nM) for 2 hours at room temperature. In a similar manner, 10 uL whole blood aliquots from *IL-1R*$^{-/-}$ or *Wt* mice were mixed with 5 uL washed SCD erythrocytes and then incubated with PBS or IL-1β (0.01 or 0.1 nM) for 2 hours at room temperature. At the end of each respective 2 hour incubation, a 5 uL aliquot of blood was then placed on a slide with 5 uL 2% sodium metabasulite, a cover slip applied, then 3 images per slide were captured at 20x using a Nikon SE upright microscope and a Nikon DS-Fi3 camera. The percentage of sickled erythrocytes per image was quantified.

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For fractionation of cell types, 1 mL of Histopaque-1077 (Sigma, St. Louis, MO) was aliquoted for each mouse into a 5 mL round bottom tube. Whole blood from SCD, *Wt* or *IL-1R*$^{-/-}$ mice (n=4 per group) was layered on top of the Histopaque-1077, then centrifuged at 400xg 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 120xg for 10 minutes 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 8500 rpm for 10 min to pellet the platelets, then resuspended in PBS prior to treatment with PBS or IL-1β for 2 hours 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 utilizing washed SCD erythrocytes, blood was collected from SCD mice retro-orbitally under isofluorane anesthesia into 3.2% sodium citrate (9:1 blood/citrate ratio). Citrated blood was then centrifuged at 1000 rpm for 10 min, washed in PBS x 2, then resuspended in PBS at 50% hematocrit 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 IL-1β for 30 minutes or 2 hours before the platelet supernatant was applied to erythrocytes for 2 hours. In later experiments, PBS- or IL-1β- treated (30 min, 0.1 nM) platelet supernatants were incubated with 50 uM of either anti-TGF-β 1D11 antibody (Invitrogen, #16-9243-85) or control IgG antibody (Invitrogen, #16-4714-85) then added to washed SCD erythrocytes for 2 hours. Recombinant mouse TGF-β1 (R&D Systems #7666-MB, 0.025 ng/mL), apocynin (Tocris #4663, 1 mM), superoxide dismutase (SOD; Sigma #S7571-30KU, 500 U/mL), 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL; Sigma #176141, 335 uM), or the pan-PKC inhibitor Go6983 (Tocris #2285, 1 uM) was added to washed SCD erythrocytes immediately prior to 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 hours at room temperature.

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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 1000 rpm for 10 minutes. Supernatant was removed and the pelleted erythrocytes were resuspended in 150 uL RIPA buffer and stored at -80ºC until use. A calcium assay kit (Sigma #MAK022) was used according to manufacturer's directions on 50 uL of each erythrocyte lysate to measure calcium content.

Thiobarbituric acid reactive substances assay – Malondialdehyde (MDA) content of undiluted plasma from anakinra-treated animals was determined using a TBARS (TCA Method) Assay kit from Cayman chemicals (Ann Arbor, MI; #700870), following manufacturer's instructions.

Dihydroethidium (DHE) assay – Superoxide anion production from erythrocytes was determined using DHE (Invitrogen, #D11347), following 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 uM was incubated for 30 minutes with the erythrocytes while protected from light. Cells were transferred to a black, clear-bottomed 384-well plate and optimized 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).

NADPH oxidase activity assay – Erythrocytes were treated with platelet supernatant, as described above. Erythrocytes were centrifuged at 1000 rpm for 10 minutes, the supernatant was removed and cell pellets were resuspended in lysis buffer, following the protocol to assess NADPH oxidase activity in (17). The cell suspension was then dounced 50 times on ice. The homogenate protein content was measured by Pierce BCA protein assay kit (Thermo Scientific, #23225). NADPH oxidase activity was measured by a luminescence assay, as in (17), with the modification of using 10 uL homogenate and 90 uL reaction buffer rather 100 uL and 900 uL as in (17). Luminescence was monitored on Spectra Max iD5 (Molecular Devices). The among of superoxide produced was calculated by comparison with a standard curve generated using xanthine and xanthine oxidase as described by (18).

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TGF-β1 ELISA – Upon completion of sickling assay, erythrocytes were centrifuged at 1000 rpm for 10 minutes. Supernatants were removed and the pelleted erythrocytes were stored at -80ºC until use. Both preparation of lysates and ELISA process was performed according to manufacturer's instructions (Invitrogen, #BMS608-4).

Immunohistochemistry – Formalin-fixed spleens were embedded in paraffin and cut in 5 um sections before staining using anti-TGF-β 1D11 antibody (Invitrogen, #16-9243-85, 1:100). Positive stained area was detected using a biotin-conjugated secondary antibody, images were acquired on a Nikon Microphot-SA (Nikon Instruments Inc, #15941) with a MicroPublisher 3.3RTB camera (Teledyne Qimaging, #Q25984) and analyzed by Image J software (10 fields of view per mouse, 20x). Positive staining area was expressed as a percentage of the total area.

Statistical analysis – Data are represented as mean ± standard deviation. Statistical analysis was carried out using Graphpad Prism. 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 2-way 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 hemolysis and anemia in SCD mice

SCD mice were given daily i.p. injections of anakinra or vehicle for two weeks, then blood cell counts and hemolysis markers were assessed. No significant differences were observed in circulating erythrocyte, leukocyte, or platelet counts. However, free hemoglobin, AST activity, and LDH activity were found to be decreased in the plasma of mice treated with anakinra for 2 weeks (Table 1).

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1 After four weeks of treatment with anakinra, erythrocyte count, hemoglobin and
2 hematocrit were all increased compared to vehicle-treated SCD mice (Table 2).
3 Additionally, reticulocyte percentages were decreased in anakinra-treated mice (Table
4 2), suggesting reduced hemolysis. Circulating concentrations of platelets (1233 ± 74.61
5 K/uL vs 1196 ± 152.20 K/uL, p=0.58) were not different between anakinra and vehicle-
6 treated mice.

7

8 ***IL-1β promotes sickling in whole blood ex vivo***

9 To test whether the improved anemia observed in anakinra-treated mice was
10 associated with reduced erythrocyte sickling, a sickling assay was performed with whole
11 blood from treated SCD mice using sodium metabisulfite, a reducing agent which
12 promotes deoxygenation in sickle erythrocytes by scavenging oxygen (14, 15). The
13 percent of sickled erythrocytes was decreased in blood from anakinra-treated mice
14 compared to controls (Figure 1A-C). Next, whole blood from untreated SCD mice was
15 incubated for ten minutes with PBS or anakinra, before the addition of IL-1β (0, 0.01, or
16 0.10 nM IL-1β, final concentration). A dose-related increase in sickling was observed in
17 samples pre-treated with PBS while this was blocked in samples pre-treated with
18 anakinra (Figure 1D). To further verify the specificity of the sickling response to IL-1β,
19 whole blood was collected from WT or *IL-1R*$^{-/-}$ mice, to which washed SCD erythrocytes
20 were added. Basal sickling rates were not different between SCD erythrocytes
21 incubated with *Wt* or *IL-1R*$^{-/-}$ whole blood (Figure 1E). Following addition of IL-1β a
22 dose-response increase in sickling was observed in WT blood samples, that was not
23 observed in *IL-1R*$^{-/-}$ blood samples (Figure 1E).

24

25 ***IL-1β promotes sickling indirectly through action on platelets***

26 To determine if IL-1β could directly promote *ex vivo* sickling, washed erythrocytes were
27 prepared from SCD mice and then pre-treated with PBS or anakinra for 10 minutes,
28 before the addition of PBS or IL-1β. In contrast to the previous whole blood
29 experiments, the sickling rates of washed erythrocytes were unresponsive to IL-1β or

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1 anakinra (Figure 2). This suggests that IL-1β indirectly promotes sickling by acting on a
2 separate cell population. Therefore, to determine the relevant cellular pool for IL-1β
3 effects on *ex vivo* sickling, cell fractions were prepared from SCD blood using
4 Histopaque-1077, and then treated with PBS or IL-1β. After treatment, cells were
5 centrifuged and the supernatants were applied to washed SCD erythrocytes. As shown
6 in Figure 2, erythrocytes incubated without supernatant or with supernatant from
7 polymorphonuclear or mononuclear cells did not respond to IL-1β. However,
8 supernatants from platelet cell fraction showed a dose- response increase in sickling
9 when applied to washed SCD erythrocytes (Figure 2).

10 The platelets of sickle cell patients are functionally hyperactive (19, 20). To further
11 delineate if the platelet-mediated ability to induce sickling in response to IL-1β was
12 unique to SCD platelets, the same experiment was performed with WT cell fractions.
13 similar to SCD cell fractions, supernatants prepared from IL-1β-treated mononuclear
14 cells did not produce increased sickling when incubated with SCD erythrocytes (Table
15 3). However, supernatants from IL-1β-treated platelets did promote the dose-response
16 increase in sickling, which was not observed in IL-1β-treated platelet supernatants from
17 *IL-1R$^{-/-}$* mice (Table 3). Therefore, the release of the sickling factor from platelets in
18 response to IL-1β is dependent on IL1R presence and is not a unique response of SCD
19 platelets.

20

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

22 To gain insight into the kinetics of the response of platelets to IL-1β, platelets were
23 incubated with IL-1β for 30 minutes or 2 hours before the platelet supernatant was
24 applied to erythrocytes for 2 hours. The supernatant from platelets incubated with IL-1β
25 for 30 minutes was sufficient to generate a similar sickling response as a 2 hour IL-1β
26 incubation (**Table 4**).

27

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

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Reactive oxygen species have been shown to promote erythrocyte membrane rigidity (21), mechanical instability (22), and to oxidize hemoglobin (22, 23). Treatment of sickle RBCs with antioxidants can prevent irreversible sickling *in vitro* (24). We therefore hypothesized 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 following incubation with the IL-1β-treated platelet supernatant relative to PBS-treated samples (Figure 3). Apocynin, an inhibitor of superoxide generation by NADPH oxidase (NOX), and Tempol, a superoxide dismutase (SOD) mimetic, were used as negative controls to verify specificity of superoxide assessment. Inhibition of IL1R1 with anakinra *in vivo* also decreased plasma ROS as assessed by plasma malondialdehyde (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 (Figure 3).

Platelet supernatant-mediated sickling is abrogated by treatment with anti-TGFβ 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 hypothesized 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 (Figure 4A) but not by an anti-IgG control antibody (Figure 4A). The inclusion of recombinant TGF-β increased sickling relative to PBS-treated platelet supernatants, to a similar percentage as IL-1β-treated supernatants (Figure 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

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of erythrocytes with anti-TGF-β antibody and apocynin did not further decrease sickling relative to each treatment alone (Figure 4A). IL-1β-treated platelet supernatant induced an increase in NOX activity in erythrocytes after 2 hour treatment relative to controls (Figure 4B). To further verify the reliance of IL-1β-mediated sickling on superoxide generation, superoxide dismutase (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 (Figure 4C). Finally, the increase in sickling observed with recombinant TGF-β incubation was abrogated by apocynin co-treatment (Figure 4C).

To verify the transfer of TGF-β to erythrocytes, the relative concentration of TGF-β present in washed erythrocytes post-incubation with IL-1β treated platelet supernatants was assessed via ELISA. TGF-β concentration was increased in erythrocyte lysates which had received IL-1β-treated platelet supernatant relative to controls (Figure 5a). However, in plasma from mice treated 2 weeks with anakinra, the plasma concentration of TGF-β was not decreased relative to vehicle treated mice as assessed by ELISA (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 (Figure 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 uM, as previously (28)) was observed to abrogate the sickling response of IL-1β treated platelet supernatants (Figure 5c). In support of this mechanism, intracellular calcium was also observed to increased significantly in erythrocyte lysates following incubation with IL-1β treated platelet supernatants (Figure 5d).

Discussion

Hemolysis promotes inflammasome activation with increased IL-1β production (29). Subsequent activation of IL-1β signaling pathways may contribute to organ damage and

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perpetuate hemolysis in chronic hemolytic syndromes such as SCD (29, 30). IL-1β signals through the IL-1R to trigger inflammatory responses which are mediated by multiple cell types (31-34). Some limited evidence for a causal role for IL-1β signaling 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-hematopoietic cellular pools has shown to be beneficial in reducing hemolysis-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-hematopoietic IL-1R deficiency state, no effect was observed on anemic parameters (6). To determine the effects of global IL-1R inhibition on anemia in SCD, mice were treated for several weeks with anakinra in the current study. After 2 weeks of treatment, no difference in anemic parameters (HB, HCT, RBC) were observed between mice treated with anakinra or vehicle control, however circulating levels of serum AST, LDH, and cell-free hemoglobin were reduced in anakinra-treated mice, suggesting reduced hemolysis. Following 4 weeks of treatment, anemia was improved in anakinra-treated mice while markers of hemolysis remained reduced indicating global IL-1R antagonism might improve anemia via reduced hemolysis in SCD. Since non-hematopoietic genetic deficiency of IL-1R did not affect anemia in SCD (6), these findings suggest hematopoietic expression of IL-1R promotes hemolysis and anemia 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 hemolysis observed *in vivo* following anakinra treatment, erythrocyte sickling in whole blood was reduced 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 $IL\text{-}1R^{+/+}$ or $IL\text{-}1R^{-/-}$ mice, a dose-dependent effect of exogenous IL-1β was

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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. Following 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 anemia. 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 which 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 signaling 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 anemia through the signaling 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 minutes of IL-1β stimulation was not addressed in this study.

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Platelets release TGF-β and platelet-generated TGF-β contributes largely to TGF-β plasma levels (41). A neutralizing 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 this study is capable of inhibiting all 3 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 this study, erythrocyte-associated TGF-β was observed to increase post-treatment with stimulated platelet supernatant, however it is possible that platelets may respond to IL-1β with the release of other factors which 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 hemoglobin 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

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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 signaling 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 this study include pharmacologic antagonism of candidate cytokines/receptors, which may have off-target effects *in vivo*. Additionally, *in vitro* sickling assays were used to explore cell types and downstream pathways related to the IL-1 receptor 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 endpoints in murine models of sickle cell disease. Additionally, while the excitation and emission wavelengths used to detect superoxide were optimized 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. Leukocyte-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 reactive oxygen species (ROS) generation (51, 52), which could also promote erythrocyte sickling in SCD through membrane oxidative injury (5).

In conclusion, pharmacologic antagonism of the IL-1R in SCD mice improves anemia. 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, such as platelet-specific TGF-β null mutants,

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1 will be necessary to confirm the *in vivo* significance of these findings. The clinical
2 implications are that short-term treatment strategies targeting IL-1R, TGF-β or
3 downstream effector molecules may be beneficial in managing complications of SCD.
4

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21 **Tables**

22 **Table 1. Markers of hemolysis**

	PBS	Anakinra	*p value*
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**

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1 Table 1. The average ± standard deviation for each marker of hemolysis is indicated in
2 the table (n = 4 per group) for SCD mice after 2 weeks of anakinra treatment (10 mg/kg,
3 i.p. daily). HB: hemoglobin; LDH: lactate dehydrogenase, AST: aspartate
4 aminotransferase.

5

6 **Table 2. Effect of Anakinra treatment on anemia**

	PBS	Anakinra	*p value*
HB (g/dL)	7.72 ± 0.13	9.8 ± 0.86	*0.030**
HCT (%)	32.06 ± 0.93	46.25 ± 4.03	*0.040**
RBC (M/uL)	6.77 ± 0.18	9.01 ± 0.78	*0.048**
Retic (%)	32.45 ± 2.74	25.31 ± 1.79	*0.018**

7 Table 2. The average ± standard deviation is indicated in the table (n = 4 per group) for
8 SCD mice after 4 weeks of anakinra treatment (10 mg/kg, i.p. daily). HB: hemoglobin;
9 HCT: hematocrit; RBC: red blood cell; Retic: reticulocyte.

10

11 **Table 3. *In vitro* sickling assays with washed RBCs and fractionated cell**
12 **supernatants**

	Treatment w IL-1β (nM)			p values	
	0	0.01	0.1	0 to 0.01	0 to 0.1
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****
$IL1R^{-/-}$ MN supt	9.20 ± 2.52	12.44 ± 2.46	11.11 ± 0.68	*0.4930*	*0.2660*

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IL1R$^{-/-}$ PLT supt	11.79 ± 0.89	11.39 ± 2.65	12.64 ± 4.64	*0.8380*	*0.7090*

1 Table 3. Whole blood was fractionated into different cell populations. The isolated cell
2 populations were treated for 2 hours with indicated concentrations of mouse
3 recombinant IL-1β before centrifugation to remove cells from supernatants. The
4 supernatants were applied to washed SCD RBCs and incubated for 2 hours, after which
5 time sickling was assessed. The average ± standard deviation of the percentage of
6 sickled RBCs is indicated in the table (n = 3 per group). Significance was determined
7 by a one-way ANOVA, followed by a post-hoc analysis with Turkey's multiple
8 comparisons tests. Supt: supernatant; PMN: polymorphonuclear cells; MN:
9 Mononuclear cells; PLT: platelet.

10

11 **Table 4. *In vitro* sickling assays for 30 minutes or 2 hour incubation with platelet**
12 **supernatants**

	PBS	IL-1β (0.1 nM)	*p value*
30 minutes	10.73 ± 0.82	17.19 ± 1.04	*0.001***
2 hours	11.15 ± 1.08	18.38 ± 1.75	*0.009***

13

14 **Table 4.** Isolated platelets were treated for 30 minutes or 2 hours with indicated
15 concentrations of mouse recombinant IL-1β before centrifugation to remove cells from
16 supernatants. The supernatants were applied to washed SCD RBCs and incubated for
17 2 hours, after which time sickling was assessed. The average ± standard deviation of
18 the percentage of sickled RBCs is indicated in the table (n = 3 per group). Significance
19 was determined by a one-way ANOVA, followed by a post-hoc analysis with Turkey's
20 multiple comparisons tests.

21

22 **Figure Legends**

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1 **Figure 1. Whole blood sickling assays with Anakinra. A)** Mice were given daily i.p.
2 injections with vehicle (PBS) or Anakinra for 4 weeks. The percentage of sickled cells
3 were quantified (3 fields of view per mouse, n=4 per group) and the mean ± standard
4 deviation is shown; significance determined by an unpaired student's t-test.
5 Representative images of blood cells from PBS-treated mice **(B)** and Anakinra-treated
6 mice **(C)** are shown. Arrows indicate sickled cells. **D)** Whole blood from untreated SCD
7 mice was drawn and treated *ex vivo* with ± anakinra ± IL-1β before the percentage of
8 sickled cells was assessed. Mean ± standard deviation is shown (n=4). Significance
9 was determined by an independent 2-way ANOVA, followed by a post-hoc analysis with
10 Turkey's and Sidak's multiple comparisons tests. **E)** Washed SCD RBCs were
11 incubated with whole blood from either WT or IL-1R$^{-/-}$ mice at a ratio of 1:2 in the
12 presence of varied concentrations of IL-1β before assessment of sickling. The mean of
13 the percentage of sickled cells ± standard deviation is shown. Significance was
14 determined by an independent 2-way ANOVA, followed by a post-hoc analysis with
15 Turkey's and Sidak's multiple comparisons tests. "n.s." = not significant, *= p<0.05, ** =
16 p<0.01, *** = p< 0.005, **** = p <0.0001.

17 **Figure 2. IL-1β indirectly promotes *in vitro* sickling. A)** Washed SCD RBCs were
18 incubated with ± anakinra (149.25 nM) for 10 min before incubation ± IL-1β (0, 0.01, 0.1
19 nM) for 2 hours. Then, the percentage of sickled cells was assessed. Mean ± standard
20 deviation is shown (n=4) and is not significantly different. **B)** Cell fractions were
21 collected, incubated ± IL-1β (0, 0.01, 0.1 nM) for 2 hours, and then treated supernatants
22 were applied to washed SCD RBCs for another 2 hours before the percentage of
23 sickled cells was assessed. Significance was determined by an independent 2-way
24 ANOVA, followed by a post-hoc analysis with Turkey's and Sidak's multiple
25 comparisons tests. "n.s." = not significant, *= p<0.05, ** = p<0.01, *** = p< 0.005, **** =
26 p <0.0001.

27 **Figure 3. Alterations to reactive oxygen species in response to IL-1β or Anakinra**
28 **A)** DHE at the concentration of 10 uM was incubated for 30 minutes with the
29 erythrocytes while protected from light. Cells were transferred to a black, clear-bottomed
30 384-well plate and the optimized detection of the superoxide specific product 2-OH-

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ethidium (Ex/Em: 405/570) over the unspecific product ethidium was obtained. Mean ± standard deviation is shown (n=3 per group). Significance was determined by an independent 2-way ANOVA, followed by a post-hoc analysis with Turkey's and Sidak's multiple comparisons tests. **B)** Mean plasma MDA concentrations ± standard deviation are graphed from mice receiving anakinra for 2 weeks or 4 weeks, n=4 each group. Significance was determined with student's t-test, *= $p<0.05$, ** = $p<0.01$, *** = $p<0.005$.

Figure 4. Perturbation of IL-1β-mediated sickling with anti-TGF-β antibody, NOX inhibition, or ROS scavengers. A) Washed SCD RBCs ± inhibitory antibodies, recombinant TGF-β, or apocynin were incubated for 2 hours with supernatants from platelets treated for 30 minutes ± IL-1β. The mean ± standard deviation of the percentage of sickled cells is shown; each dot indicates the percentage sickled cells for a specific field of view (2-3 fields of view per mouse, n=3 per group). **** = $p <0.0001$ relative to 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 hours with supernatants from platelets treated for 30 minutes ± IL-1β, then erythrocytes were lysed on ice and the lysates were tested for NOX activity. The mean ± standard deviation of superoxide anion (nM) generated per ug protein is shown; 3 technical replicates per mouse, n=5 mice). Significance was determined using student's t-test, $p<0.05$. **C)** Washed SCD RBCs ± Tempol, SOD, apocynin or recombinant TGF-β were incubated for 2 hours with supernatants from platelets treated for 30 minutes ± IL-1β. The mean ± standard deviation of the percentage of sickled cells is shown; each dot indicates the percentage sickled cells for a specific field of view (3 fields of view per mouse, n=3 per group). Significance was determined by an independent 2-way 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 IL1β-treated platelet supernatant.

Figure 5. TGF-β content downstream of IL-1β manipulation and involvement of PKC in the proposed mechanism. A) ELISA of total TGF-β concentration ± standard deviation of erythrocyte lysates collected after a 2 hour incubation with supernatants

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from platelets treated for 30 minutes ± IL-1β (n= 4 per 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 percent splenic area positive for TGF-β ± standard deviation, n=4 per group. Significance was determined by student's t-test, * = p<0.05. **C)** Sickling assay including pan-PKC inhibition with G06983 (1uM). Significance was determined by an independent 2-way 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. **D)** Calcium concentrations of erythrocyte lysates after incubation with platelet supernatants ± IL-1β for 2 hours (n=4 per group). Significance was determined with student's t-test, ** = p<0.01.

Figure 6. Proposed model for IL-1β-mediated increase in sickling. In response to IL-1β stimulation, platelets release active TGF-β among other possible factors. TGF-β released from IL-1β-treated platelets leads to activation of erythrocyte PKC which further activates erythrocyte NOX. The superoxide ($O_2^{\cdot -}$) produced by activated NOX, and the hydrogen peroxide (H_2O_2) produced from $O_2^{\cdot -}$, promote erythrocyte sickling. The observed increase in calcium may promote PKC activity and independently promote erythrocyte sickling.

Figures

Figure 1. Whole blood sickling assays with Anakinra

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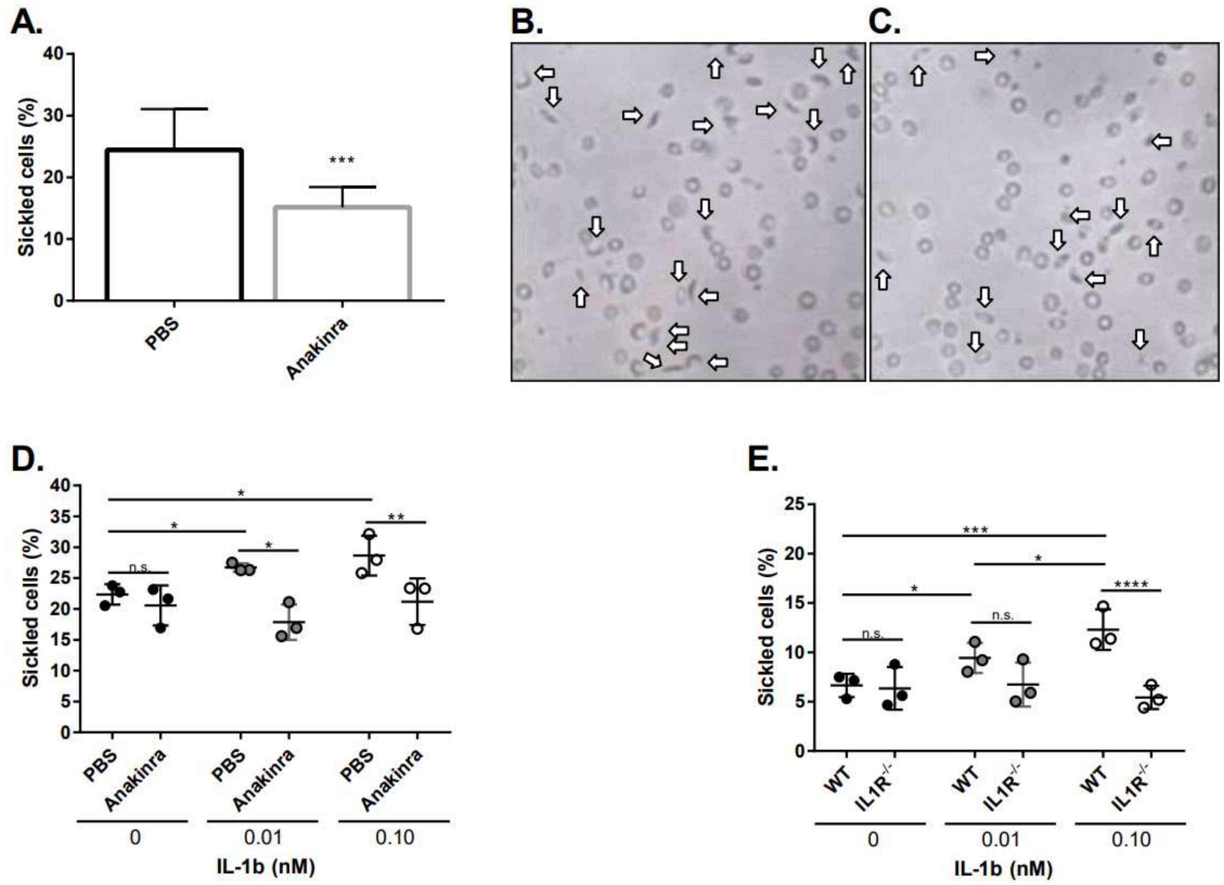

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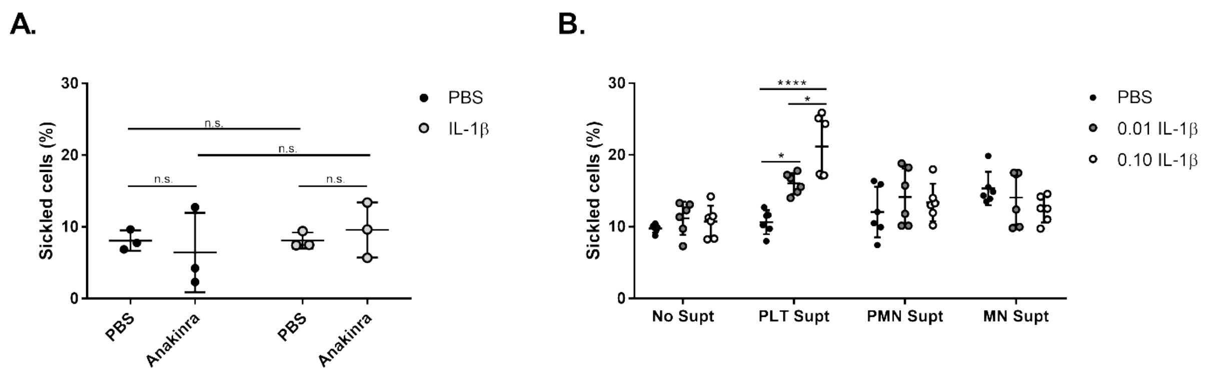
5 Figure 2. IL-1β indirectly promotes *in vitro* sickling.



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2 Figure 3. Alterations to reactive oxygen species in response to IL-1β or Anakinra

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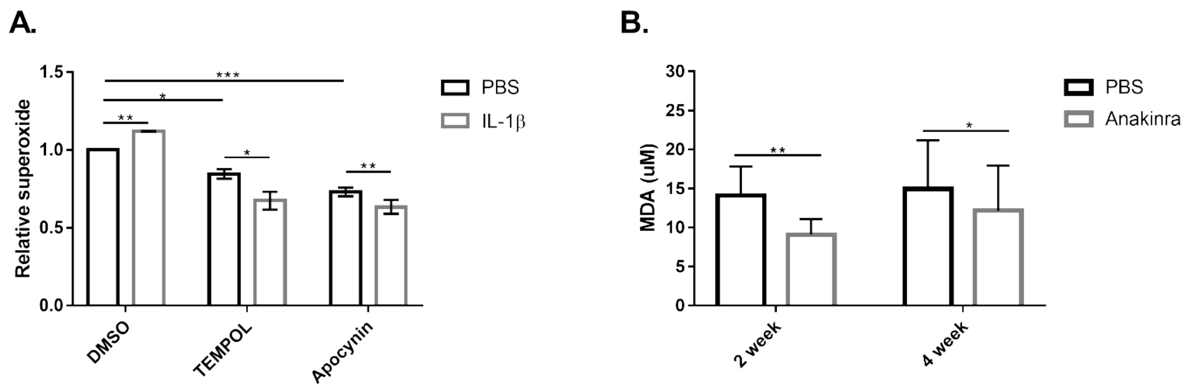

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5 Figure 4. Perturbation of IL-1β-mediated sickling with anti-TGF-β antibody, NOX

6 inhibition, or ROS scavengers

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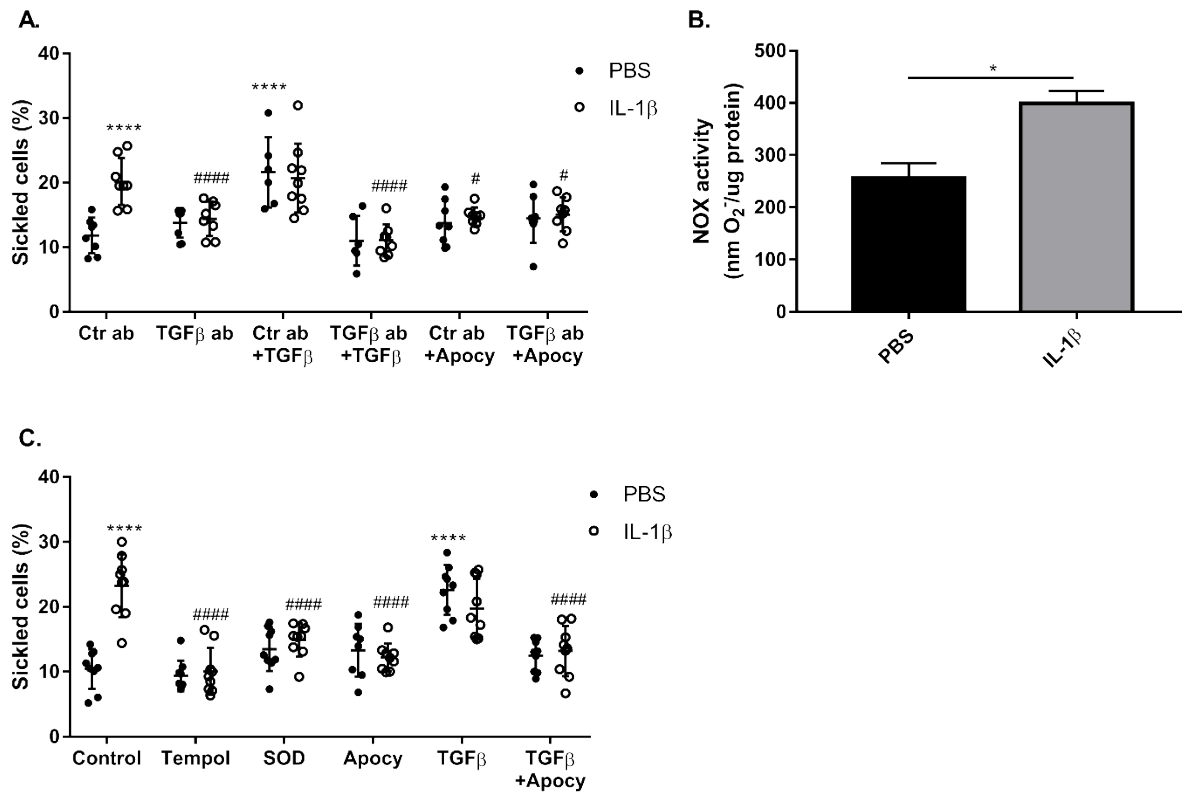

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2 Figure 5. TGF-β content downstream of IL-1β manipulation and involvement of PKC in

3 the proposed mechanism.

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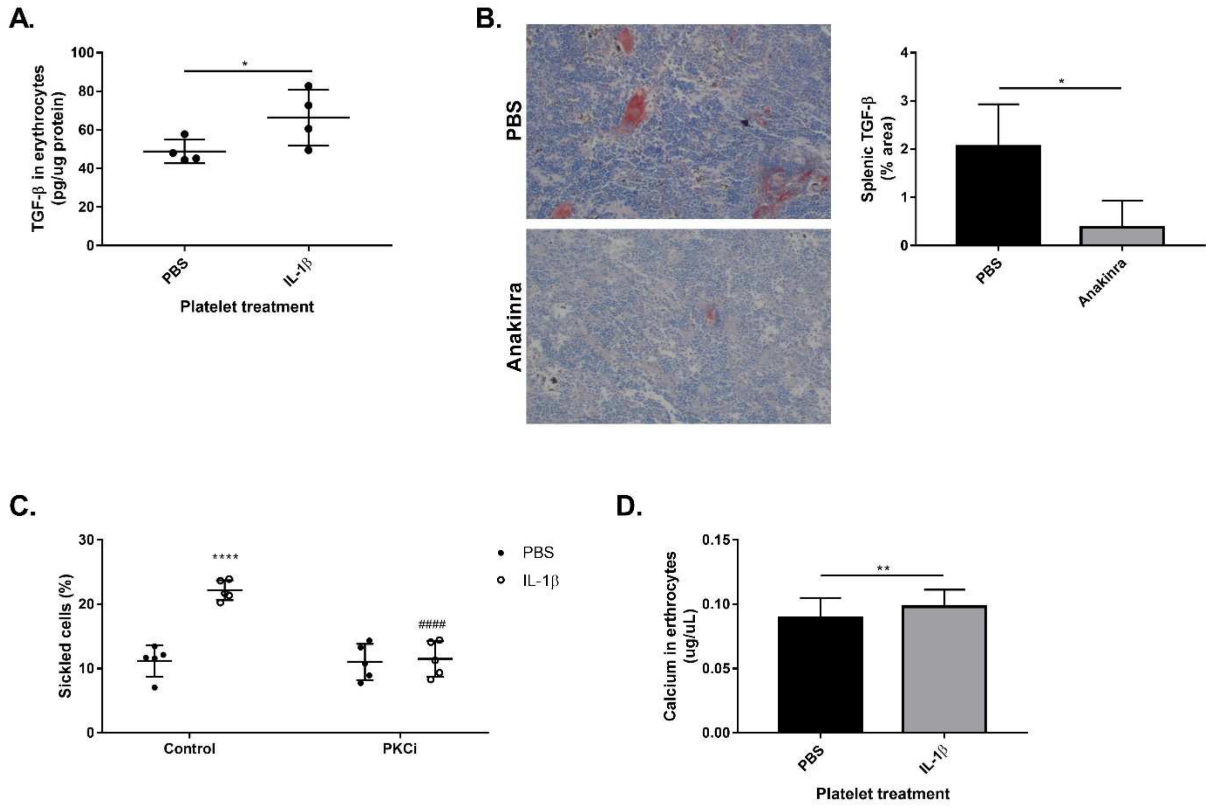
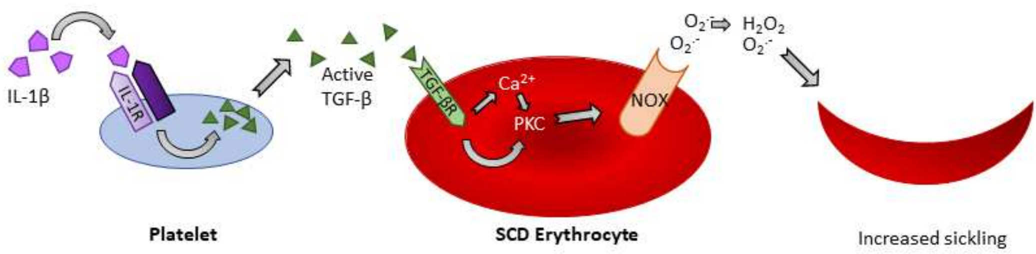


2 **Figure 6. Proposed model for IL-1β-mediated increase in sickling**



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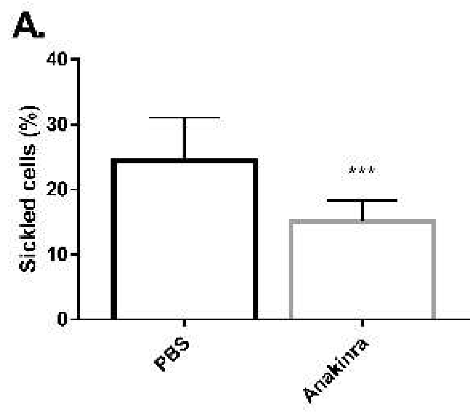

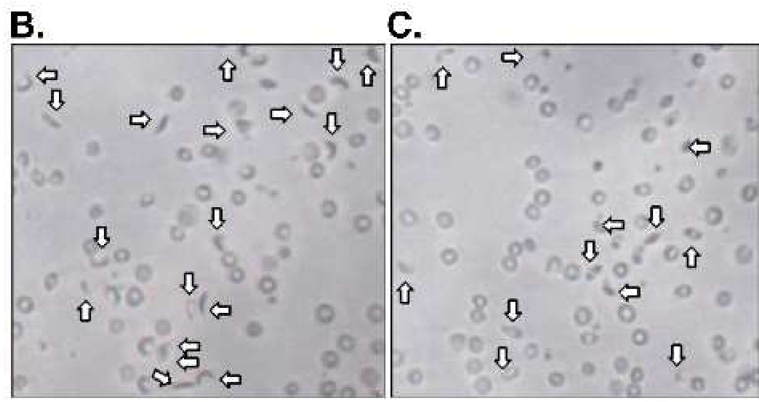

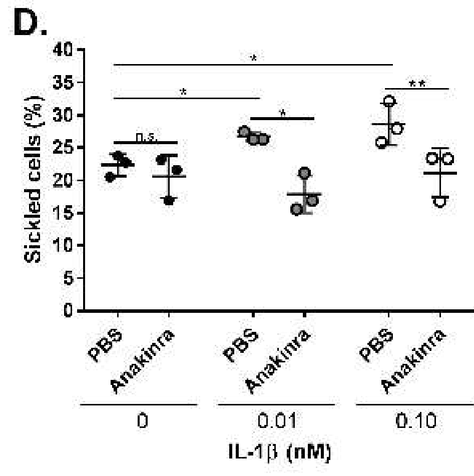

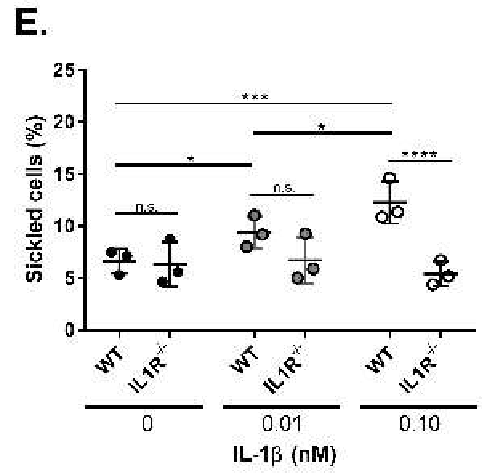

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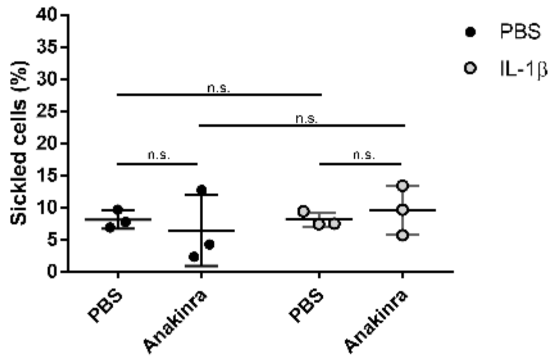

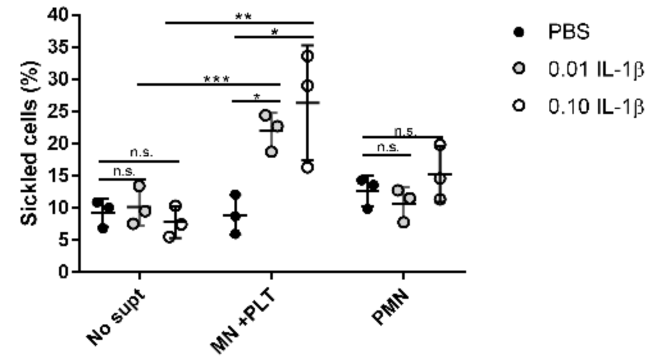

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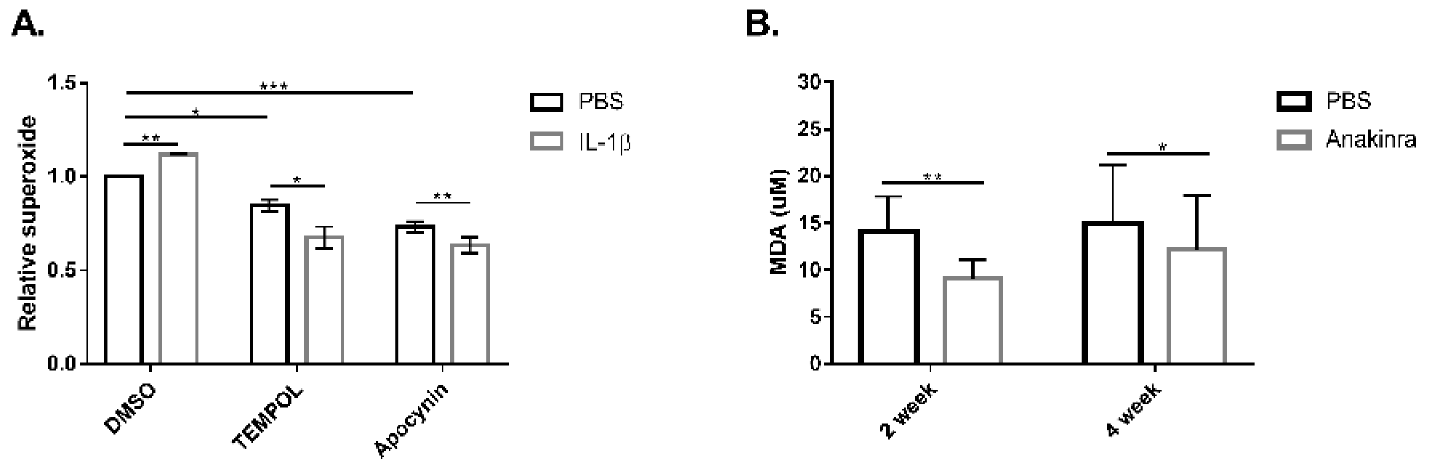

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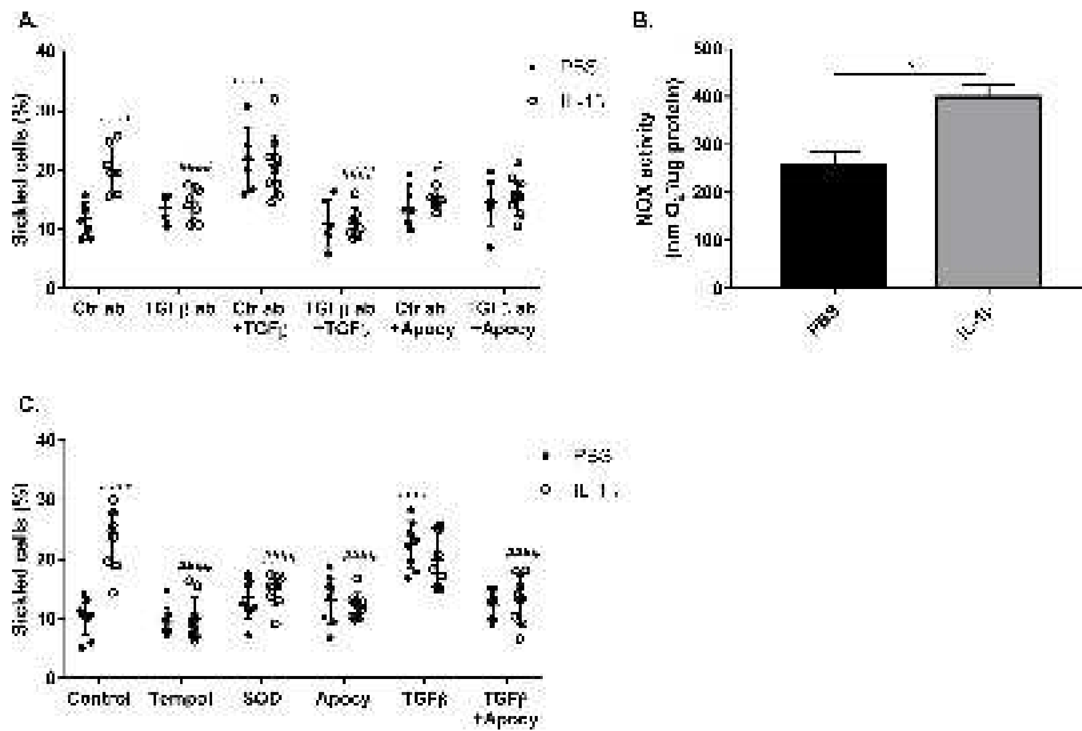

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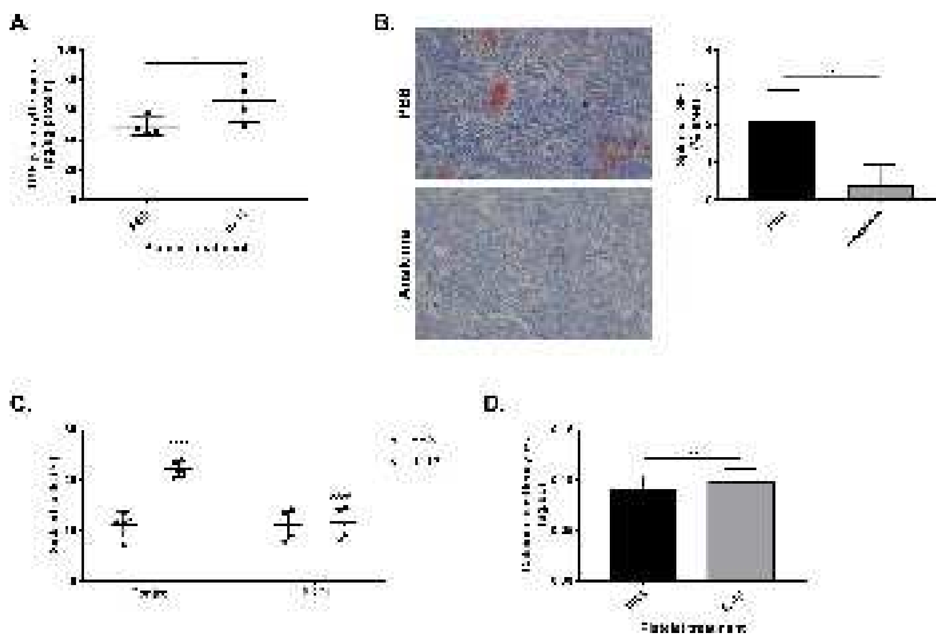

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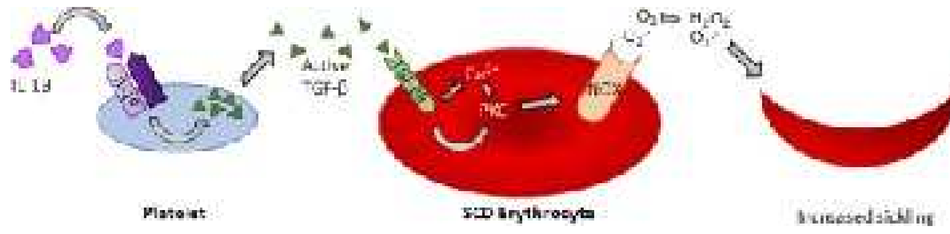

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