Chronic Inflammation in Sickle Cell Disease: Potential Role of Platelets and the Inflammatory Mediator CD40L

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ABSTRACT

SHERITHA P. LEE: Chronic Inflammation in Sickle Cell Disease: Potential Role of Platelets and the Inflammatory Mediator CD40L
(Under the direction of Leslie V. Parise, PhD)

Chronic inflammation is a poorly understood, but problematic, manisfestation of sickle cell disease (SCD). The potent inflammatory mediator CD40L increases leukocyte proliferation, endothelial adhesiveness and procoagulant activity once it is exposed and released from encrypted sites following platelet activation. Since elevated leukocyte counts, adhesion and coagulation are associated with more clinically severe courses of SCD along with platelets that are more activated than normal, platelet CD40L is hypothesized to be an important mediator of inflammation in SCD. Data shown here indicate that CD40L is thirty-fold higher in SCD plasma but significantly reduced in SCD platelets. SCD plasma induces B cell proliferation, endothelial activation and procoagulant tissue factor production, all in a manner partially dependent on recognition of CD40L. When CD40L activity was blocked in a mouse model of SCD, lung, liver and kidney pathology was demonstrably reduced. More dramatically, anti-CD40L treatment of SCD mice prevented the architectural disruption and drastic enlargement of the spleen that is characteristic of SCD. These data suggest that functional CD40L is available and likely mediates inflammation in SCD. Furthermore, in a phase I clinical trial, the αIIbβ3 antagonist eptifibatide reduced plasma CD40L, favorably altered the inflammatory cytokine profile of SCD plasma and was well-tolerated by SCD patients. This study provides the first evidence that inflammation and organ pathology in SCD are mediated by platelet-derived CD40L. Since plasma CD40L can be safely reduced by anti-platelet medication, we identify both CD40L and platelets as potential therapeutic targets to treat SCD.

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LIST OF ABBREVIATIONS

5–HT	serotonin
βTG	beta-thromboglobulin
ACS	acute coronary syndrome
ADP	adenosine diphosphate
BMT	bone marrow transplant
CD40L	CD40 ligand
DLAM	Department of Laboratory and Animal Medicine
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GCRC	General Clinical Research Center
GP	glycoprotein
H&E	hematoxylin and eosin
Hb	hemoglobin
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IACUC	Institutional Animal Care and Use Committee
ICAM-1	intercellular adhesion molecule 1
IgG	gamma immunoglobulin
MFI	mean fluorescence intensity
MIP-1α	Macrophage inhibitory protein -1α
MMP	matrix metalloproteinase
PF4	platelet factor 4
PFP	platelet and microparticle-free plasma
PGI_2	Prostaglandin I_2
PTAH	phosphotungstic acid hematoxylin
RBC	red blood cells
SA	hemoglobin S and A heterozygote
SCD	sickle cell disease
sCD40L	soluble CD40 ligand
SLE	systemic lupus erythematous
SS	hemoglobin S homozygote
TF	tissue factor
TNFα	tumor necrosis factor α
TRAP	thrombin receptor activating peptide
VCAM-1	vascular cell adhesion molecule 1
WBC	white blood cells
WT	wild-type

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Historical Overview

Sickle cell disease (SCD) can be traced back as far as 1670 in 9 successive generations of at least one Ghanaian family.[1] Long-standing beliefs and practices of the Yoruba and Ibo of Nigeria suggest that Africans may have had an awareness of SCD that predates oral and written histories.[2,3] The transport of millions of Africans via the Atlantic slave trade led to the dispersal of the sickle gene and SCD throughout the Americas.[4] The disease first came to the attention of Western medicine in 1910 when the blood cells of an anemic West Indian student were described as abnormally sickle-shaped.[5]

Later studies showed that sickling is reversible[6] and occurs in response to a decrease in oxygen,[7] suggesting the involvement of hemoglobin (Hb). The relationship became clearer when Hb from SCD patients, like the red cells, was observed to aggregate upon deoxygenation.[8] The differential electrophoretic mobility of normal and sickle Hb shown in 1949[9] led to the theory that the ordered structure of the abnormal Hb causes sickling,[9] making SCD the first molecular disease to be identified. The substitution of valine for glutamic acid in the sixth amino acid position of the Hb β chain was reported in 1956.[10]

Since that time, SCD has been the model disease for many fields of biomedical as well as social science. It was the first disease to be attributed to a mutation of a specific protein[10], the first disease to be shown to exhibit Mendelian properties of inheritance[11], and the landmark disease by which many racial and sociopolitical precedents have been set. The study of SCD has attracted many of the best and brightest scientists of the twentieth century. However, despite the knowledge that has amassed, not enough has been translated into effective treatment for the many people around the world who still suffer with this disease. Widespread use of hydroxyurea has resulted in fewer and shorter pain episodes as well as decreased incidences of acute chest syndrome[12] for those who can tolerate the drug, but toxicity puts limitations on its use. Stem cell transplantation is also promising; however, high risk and limited access prevent this treatment from being the panacea many thought it would be. We are still waiting for the gene therapy that will cure the disease and/or the miracle drug that will make pain episodes a thing of the past. Until then, the approaching one-hundredth year anniversary of SCD research marks a time filled with both disappointment and promise. Sickle cell patients are living longer, more productive lives; but every new finding, every insight gained, is still needed to put an end to the suffering.

Sickle cell disease is a chronic inflammatory condition.

Sickle cell disease is a multi-factorial syndrome. Hemoglobin polymerization leads to abnormally rigid and adhesive red blood cells (RBC) that obstruct blood vessels, leading to tissue damage from poor perfusion.[13] Recent reports have begun to describe sickle RBC as irritants that provoke inflammation[14] as they stimulate and damage surrounding tissues and

cells. Therefore, normal cellular responses to these stimuli become pathogenic and chronically inflammatory in the abnormal context created by the mutant Hb S. In fact, polymorphic differences in genes involved in inflammation are increasingly being thought of as reasons for the varying clinical courses of SCD patients.[15] Inflammation exacerbates vascular occlusion in SCD because of mutual feedback loops by which inflammation and coagulation are intertwined.[16]

Multiple manifestations of inflammation exist in SCD.

The classic definition of inflammation is an elevation in leukocyte counts. Patients with SCD have an abnormally high leukocyte count even in the absence of infection[17]. Baseline leukocyte counts are believed to be predictive of clinically severe SCD, a risk factor for brain infarcts[18], acute chest syndrome[19], as well as early death[20]. Case reports also document that leukocyte growth factors can bring about these undesirable outcomes in SCD patients.[21] Furthermore, the activation status of leukocytes is a predictor of clinically severe SCD,[22] and intravital microscopy of SCD mice demonstrate that leukocytes may actually initiate vascular occlusion.[23] Therefore, the high leukocyte count is inextricably linked to SCD pathogenesis, both as an indicator of inflammation, but also as a causative factor.

In addition to elevated WBC counts, patients with SCD have elevated levels of the inflammatory markers C-reactive protein[24,25], tumor necrosis factor-α and interleukin-1[26-28]. Vascular cells reportedly expressing an activated phenotype in SCD include endothelial cells[29], neutrophils[30,31], monocytes[24,32] and platelets.[33,34]

Platelets likely play a role in SCD inflammation.

It is our hypothesis that platelets lie at the heart of the chronic inflammation in SCD. Virchow proposed in 1856 that endothelial injury, hemostasis, and a hypercoaguable state are the basis for thrombotic activity [35]. Our current understanding of SCD includes evidence that all three components of Virchow's triad exist in these patients with thrombosis likely at the center of the inflammatory activity. Indeed, platelets are chronically hyperactive in SCD,[34,36] and platelets increase sickle RBC adhesion to the endothelium.[37] However, the extent to which platelet activity contributes to SCD pathology or which of the many platelet functions contribute to SCD is still undefined.

Previous reports have suggested that platelets have the potential to contribute to SCD via a minimum of 5 ways:

- Platelet α granules expose P-selectin and release adhesion factors.
- Platelet dense granules release proaggregatory and inflammatory factors.
- Platelets promote procoagulant activity through initiation of the coagulation cascade.
- Platelets aggregate.
- Platelets expose and release CD40L.

Understanding platelet activation in the context of SCD may be essential for understanding and alleviating symptoms of the disease.

Platelet α granules expose P-selectin and release adhesion factors

The exposure of P-selectin to the platelet surface[38] and an increase in soluble P-selectin[39] correlate positively with pain episodes in SCD. Platelet P-selectin may serve only as a

marker of platelet activation and not have a direct role in SCD pathology;[23] however, P-selectin is known to adhere to sickle RBC[40] and may increase the aggregation of platelets to leukocytes.[41] Furthermore, platelet P-selectin may increase endothelial activation,[42] which likely contributes to vaso-occlusion due to increased sickle RBC adhesion. Platelet α granules also release the glycoproteins fibrinogen, fibronectin, vitronectin, von Willebrand factor, and thrombospondin.[43] The higher plasma fibrinogen concentration may then increase RBC adhesivity to the endothelium,[44] while the vitronectin receptor is reportedly upregulated in SCD and promotes neutrophil adhesion.[39] Fibronectin, von Willebrand factor, and thrombospondin are adhesive to sickle RBC under flow conditions.[45,46] In addition to supporting adhesion, thrombospondin increases sickle RBC adhesiveness by transmitting signals through the RBC receptor CD47, or integrin-associated protein to activate α4β1.[47] Thrombospondin and von Willebrand factor also contribute to adhesion by acting as bridges between sickle RBC and the endothelium[48-50] and von Willebrand factor may link platelets to the endothelium.[51] Therefore, platelet α granules may contribute to SCD by releasing factors that upregulate adhesion.

Platelet dense granules release pro-aggregatory and proinflammatory factors

Upon activation, platelet dense granules can contribute to increased platelet aggregation by releasing adenine nucleotides, inflammatory factors, and bivalent cations.[43] Adenosine diphosphate (ADP) stimulation of purinergic receptors is a major mechanism of platelet activation.[52] Therefore, the release of ADP from platelet dense granules plays a critical role in hemostasis by recruiting other platelets and perpetuating platelet activation.[52] Since the platelets of SCD patients in crisis are depleted of ADP stores,[53] ADP may increase SCD

pathology via this mechanism. Dense granules may also contribute to SCD pathology through the release of inflammatory factors. Serotonin (5-HT) is a major component of dense granules,[43] and can exacerbate SCD through its ability to cause vasoconstriction,[54] augment the activity of other vasoconstrictors,[55] and induce platelet aggregation.[56] Serotonin may also activate and recruit neutrophils[57] that may contribute to vaso-occlusion. Furthermore, 5-HT may promote coagulation[58] in a tissue factor-dependent manner.[59] Dense granules also contain and release the inflammatory mediator histamine, which recruits leukocytes[57,60] These dense granule components could participate in SCD pathology by increasing coagulation, slowing blood flow and promoting vaso-occlusion.

Platelets promote procoagulant activity through initiation of the coagulation cascade

Upon agonist stimulation, platelets release fibrinogen,[43] which then acts as a substrate for thrombin to cause clot formation.[61] SCD patients have higher levels of fibrinogen[62], prothrombin and thrombin-anti thrombin complexes[63], indicating increased coagulant activity. In addition, many of the clotting factors important to the coagulation cascade are also released from platelets upon agonist stimulation.[43] Factors V, VII, XI and XIII are all housed in and released from platelet α granules. Therefore, an increase in platelet activation logically increases coagulation and subsequently vascular occlusion in SCD. Furthermore, plasminogen, also released from platelets,[43] is thought to increase matrix metalloproteinase activity once it is converted to plasmin.[64] In SCD, this may be the mechanism by which endothelial cells[65] are released from the blood vessel wall to circulate and adhesive subendothelial extracellular matrix proteins[45] become exposed to flowing RBC.

Platelets aggregate

The extent to which platelet aggregation contributes to SCD pathology is unknown; however, both increased platelet aggregation[34] and platelet exhaustion from increased[66] *in vivo* activity are reported in SCD. A transient drop in platelet count that accompanies the onset of painful crises[67] suggests that platelet aggregation occurs during the initial phase of a crisis and may play an essential role in the process. An increase in circulating platelet aggregates in SCD crisis patients[68] provides further evidence that platelet aggregation may play a role in the clinical status of SCD patients. Therefore, inhibition of platelet aggregation may be beneficial to SCD patients.

Platelets expose and release CD40L

The potential role of platelets in SCD has been suggested for some time; however, the role of platelet CD40L has only recently been considered.[69,70] CD40L is a type II transmembrane protein and a member of the tumor necrosis factor superfamily.[71] Inducibly expressed on leukocyte subsets, smooth muscle, and epithelial cells, CD40L mediates a broad variety of immune and inflammatory responses[72] both in its membrane-bound form and as a soluble fragment.[73,74] CD40L is upregulated in diverse clinical settings including rheumatoid arthritis, neurological disorders, graft-vs-host disease, lung inflammation and chronic lymphocytic leukemia.[71,75,76] The findings that CD40L plays a prominent role in atherosclerosis[77] and that platelets express and release it upon activation[78] suggest the potential involvement of platelet CD40L in other inflammatory vascular diseases, including SCD. Furthermore, since the proinflammatory cytokines TNFα, IL-1, IL-4 and protein kinase C that induce CD40L expression[72] are upregulated in SCD,[79] increased CD40L

expression and activity are hypothesized to be important for chronic inflammation in SCD. Some potential mechanisms of CD40L involvement in SCD pathology are outlined.

CD40L may induce leukocyte proliferation

B lymphocyte differentiation and proliferation are the original functions attributed to CD40L early after discovery of the protein.[75,80] Subsequently, CD40L was shown to also regulate leukocyte counts by rescuing monocytes and dendritic cells from apoptosis.[72] Either function of CD40L might explain the elevated leukocyte counts observed in SCD patients.[13]

CD40L may increase cell adhesion by upregulating adhesion molecules and extracellular matrix exposure Lymphocytes increase ICAM and $\alpha L\beta 2$ expression in response to CD40L,[81] while endothelial cells increase P-selectin, E-selectin, VCAM and ICAM upon recognition of CD40L.[71,82] These increases in adhesion molecule expression may exacerbate SCD by increasing adhesive interactions between blood cells and the endothelia that might slow blood flow and increase the propensity of vascular occlusion. Studies in SCD mice indicate that endothelial P-selectin exposure may be critical to initiating vaso-occlusion.[23] Furthermore, CD40L-mediated alterations in matrix metalloproteinases[83] potentially explains the prevalence of circulating endothelial cells in SCD.[29] Upon exposure, extracellular matrix proteins may then support sickle RBC adhesion.[45]

CD40L may promote coagulation via an increase in tissue factor

CD40L regulates endothelial cell procoagulant activity during inflammatory responses by inducing expression of the coagulation initiator tissue factor and by downregulating thrombomodulin expression.[84] This activity is thought to be critical to plaque development in atherosclerosis[77] and may complicate SCD by contributing to the hypercoaguable state[85] of the vascular system of these patients.

CD40L upregulates production of inflammatory cytokines

Patients with SCD reportedly have increased levels of IL-1, IL-6, IL-8, and TNF.[79] These cytokines are thought to increase vaso-occlusion by increasing adhesive interactions in the vasculature of SCD patients. IL-1 and TNF activate endothelial expression of the adhesion molecule VCAM,[86,87] while IL-8 is thought to activate the VCAM receptor $\alpha 4\beta 1$ on sickle reticulocytes.[88] IL-1 and IL-6 induce secretion of plasma fibronectin[89], which can also activate and bind $\alpha 4\beta 1$.[90] Expression of all of the aforementioned cytokines can be upregulated by CD40L.[71]

Integrin $\alpha IIb\beta 3$ is essential to platelet activation and CD40L release

The integrin $\alpha IIb\beta 3$ is required for platelet release of the inflammatory mediator CD40L.[91] Upon binding to agonist, $\alpha IIb\beta 3$ participates in bi-directional signaling critical for platelet aggregation, adhesion, granule secretion, and procoagulant activity.[92] The platelets of patients lacking $\alpha IIb\beta 3$ fail to aggregate in response to agonists,[93] demonstrating the essential role that this integrin plays in platelet activity. Furthermore, blockade of $\alpha IIb\beta 3$ inhibits thrombotic vessel occlusion[94] and the prothrombin activation that leads to

coagulation.[95] As the most abundant glycoprotein on the platelet surface,[96] αIIbβ3 plays a critical role in thrombosis and hemostasis. The role of αIIbβ3 in CD40L release[91] suggests that this integrin regulates inflammation as well. Therefore, antagonism of αIIbβ3 is proposed as an effective tool to study the role of platelet activation and aggregation in SCD.

Specific Aims of the Dissertation

CD40L mediates chronic inflammation in multiple disease states.[71] This inflammatory mediator increases B cell proliferation,[75] endothelial adhesion molecule expression,[72] and procoagulant activity through upregulation of tissue factor.[97] A similar inflammatory profile is known to exist in SCD[13] but by an unknown mechanism. Platelets contain 95% of the circulating CD40L.[98] Upon activation, this CD40L is exposed to the platelet surface and released as a soluble fragment into the plasma.[99] Platelets of sickle cell disease (SCD) patients are known to be more activated than the platelets of normal individuals, suggesting CD40L might be more available and mediating chronic inflammation in these patients. However, the role of platelets and the inflammatory protein CD40L in SCD pathology is unknown. Therefore, **platelets may participate in the chronic inflammation of SCD via CD40L**. This hypothesis was tested with the following specific aims.

Aim 1. Determine if CD40L potentially contributes to SCD pathology.

In disease states where CD40L is thought to contribute to the disease process, CD40L is reportedly elevated. We measured CD40L levels in SCD patients and compared our results to findings from normal individuals. We then assessed the correlation

between elevated CD40L and the clinical state of SCD patients by comparing CD40L levels in steady state vs. crisis patients. We also determined if the endogenous CD40L in SCD patient plasma is biologically active using established CD40L functional assays. Finally, we provided evidence that platelets are the source of CD40L in SCD plasma.

Aim 2. Determine if CD40L contributes to SCD pathology *in vivo*.

The relative contribution of CD40L to SCD pathology *in vivo* was explored using a mouse model of SCD. We generated SCD mice by transplanting hematopoietic stem cells from the bone marrow of a SCD transgenic mouse into lethally irradiated recipients. Mice were then treated with an anti-CD40L antibody shown to block association of the inflammatory mediator with its receptor. Accumulated organ damage was compared in mice receiving the anti-CD40L compared with non-specific controls. The relative importance of endothelial recognition of CD40L was also assessed by imposing the SCD phenotype unto mice lacking expression of potential CD40L receptors, CD40 and β3 integrin.

Aim 3. Determine the role of platelet activation in SCD.

The role of platelets was explored in the context of SCD by blocking the major platelet integrin αIIbβ3 in human patients. Blockage of this integrin not only inhibited platelet aggregation, but also inhibited CD40L release from platelets. CD40L levels and the prevalence of platelet activation markers were assessed in patients receiving the αIIbβ3-antagonist eptifibatide. The relative changes in the

inflammatory profile of plasma from these patients was also compared before and after the eptifibatide infusion.

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CHAPTER II.

Specific Aim 1. Determine if CD40L potentially contributes to SCD pathology.

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Rationale

Chronic inflammation is one of the many manifestations of sickle cell disease (SCD).[1] Patients with SCD are prone to recurrent infections and increased leukocyte counts[2] even during the steady state. Painful crises, the major complication suffered by SCD patients, are the result of vascular occlusion[3] due to interactions of activated blood and endothelial cells.[1,4]

The polymerization of mutated SCD hemoglobin leads to red blood cell (RBC) sickling[5] and membrane anomalies[6,7] that make sickle RBC abnormally adhesive.[3,8-10] Painful vaso-occlusive crises may partly result from these adhesive RBC interacting with adhesion receptors on inflamed endothelia.[11] Indeed, RBC adhesiveness[12] and higher WBC counts[13] correlate with clinically severe SCD. However, inflammatory mediators leading to these abnormalities in SCD are not well understood.

CD40L is a TNF family member that potentially mediates inflammation in SCD. Classically known as the T cell membrane protein that induces B cell differentiation and

immunoglobulin class-switching,[14] CD40L is now known to be expressed on a variety of cell types, including platelets.[14,15] Upon activation, CD40L is exposed to the platelet surface,[15] then cleaved to generate a soluble product[16] that retains the ability to activate its widely expressed receptor CD40.[14] The CD40:CD40L interaction is thought to contribute to inflammation in systemic lupus erythematous (SLE),[17] atherosclerosis,[18] and chronic lymphocytic leukemia.[19] The soluble form of CD40L also mediates prothrombotic activity by binding to the platelet integrin glycoprotein (GP) IIb-IIIa[20] and promotes procoagulant activity through upregulation of tissue factor (TF).[21-23] Chronic inflammation, increased thrombotic activity, and hypercoagulation are known aspects of SCD.[24,25] However, the status of sCD40L in SCD is unknown. We hypothesized that platelet-derived sCD40L may be elevated in SCD as it is in other disease states and that recognition of CD40L by its receptor, CD40 may contribute to SCD pathology. However, the amount of soluble CD40L circulating in SCD patients and the role it plays in the disease has not been examined. Therefore, the goal of this aim is to determine if CD40L potentially contributes to SCD pathology.

Materials and Methods

Human Subjects

This study was approved by the UNC Committee on the Protection of the Rights of Human Subjects. Informed consent was obtained in accordance with the Declaration of Helsinki. Study subjects were hemoglobin S homozygotes aged 20 to 63 years. Patients in crisis were defined as those hospitalized for a painful vaso-occlusive event, without respect to crisis

phase. Steady state patients had not experienced a pain episode requiring acute intervention within the previous two weeks. SCD patients who had received blood transfusions or experienced symptoms not attributed to SCD within the previous two weeks were excluded, as were patients diagnosed with other inflammatory or malignant conditions. Controls were normal, healthy adults from 19 to 49 years old, and included both African-American and Caucasian subjects.

Preparation of human plasma and platelets

Peripheral blood samples were collected by venipuncture into either 0.13 M sodium citrate or ACD. Blood samples were processed within 90 minutes of blood draw. To obtain platelet- and microparticle-free plasma (PFP), samples were centrifuged at 200*g* for 15 minutes to remove red and white cells, then once at 750*g* for 20 minutes to remove platelets, and once at 16,000*g* for 20 minutes to remove microparticles. Platelet quiescence was maintained by resting samples for 15-30 minutes at 37°C before every centrifugation. Prostacyclin (5 ng/mL) was added to platelet-rich plasma prior to the second centrifugation and to platelet-poor plasma prior to the third centrifugation.

For samples used in ELISAs, PFP was defibrinated (1U/mL thrombin) as recommended by sCD40L ELISA manufacturer instructions. Plasma defibrination did not further elevate CD40L levels. Samples were stored at -80°C as per manufacturer's instructions until use in sCD40L (Alexis Biochemicals, San Diego, CA) and TF (American Diagnostica, Greenwich, CT) ELISAs.

Platelet CD40L levels

To measure platelet CD40L levels, platelet pellets were collected following a 20-minute centrifugation at 750*g*. Platelets were carefully resuspended in a modified Tyrode's buffer of 5 mM HEPES, 135 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 1 mg/ml dextrose, 1 mg/ml BSA, 50 U/ml heparin, and 10 μg/ml apyrase, pH 6.5. Platelets were standardized to $3x10^8$ platelets/ml following counting in a Beckman Coulter counter, then lysed in 10 mM CHAPS, 0.5% deoxycholate, 137 mM NaCl, 20 mM HEPES, and a broad-spectrum protease inhibitor cocktail (CalBiochem, La Jolla, CA). Concentrations of solubilized CD40L in the resulting lysates were then determined by ELISA as above.

B cell proliferation

Ramos B cells were maintained in RPMI-1640 + 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. For proliferation experiments, B cells were incubated for 72 hours in RPMI-1640 + 10% human plasma prepared as indicated above, without defibrination. sCD40L was precipitated from samples by incubating plasma with glutathione sepharose beads (Amersham Biosciences) and either anti-CD40L or non-specific control rabbit IgG overnight at 4ºC. ^{3}H-thymidine (1U/mL of culture media) was added during the last 18 hours of culture.

Monocyte tissue factor production

THP-1 monocytic cells were grown in RPMI-1640 + HEPES + 10% FBS at 37°C in 5% CO_2. For TF induction experiments, THP-1 cells were incubated in 10% human plasma that was

prepared as described above, but without defibrination. Following addition of either anti-CD40 (ATCC#HB-11339) or control antibody, THP-1 cells were incubated for 24 hours, then washed 3X in PBS before being lysed with 1% NP-40 in a Tris-based lysis buffer containing 150 mM NaCl and 2 mM EDTA, pH 7.5.

Endothelial cell isolation and culture

Collagenase (1 mg/ml) was used to harvest human umbilical vein endothelial cells (HUVEC) from the cannulated vein of human umbilical cords as described.[26] HUVEC were then cultured in M199 media with 20% FBS, heparin, non-essential amino acids, endothelial cell growth factor, penicillin and streptomycin. HUVEC were used from passages 2-4.

Cell surface ELISA

HUVEC were grown to over-confluence to induce quiescence in 12-well tissue culture dishes, then treated with 10% human plasma for 24 hours. Plasma was prepared as above, but was not defibrinated. For inhibition experiments, rabbit anti-human CD40 polyclonal antibody (Research Diagnostics, Flanders, NJ) or rabbit IgG were added 30 minutes prior to treatment with human plasma. Cells were washed 3X with PBS, fixed with 1% paraformaldehyde, then blocked with 10% non-fat dry milk. A monoclonal antibody against ICAM-1 (Serotec, Inc., Raleigh, NC) at 1 μg/ml was incubated overnight at 4°C, followed by an anti-mouse IgG:horseradish-peroxidase conjugate, with 3X PBS + 0.1% Tween-20 washes between each step. Reactions were developed in tetramethylbenzidine (Sigma) and stopped with 2N H_2SO_4. Absorbance was read at 450 nm.

Statistical Analyses

All data are presented as mean ± standard deviation. Levels of soluble CD40L in human plasma were compared using nonparametric Wilcoxon sum rank tests as computed by SAS Proc Npar1way where average scores were used as ties. The student's *t* test was used to determine significance for all other data, at a power of 0.1.

Results

CD40L is elevated in the plasma of SCD patients

To determine if CD40L is elevated in SCD, sCD40L levels were measured by ELISA in platelet- and microparticle-free plasma (PFP). PFP was prepared by centrifugation of quiescent platelet-rich plasma under conditions determined to prevent platelet activation or contribution of microparticles, as described in Methods. The average concentration of sCD40L in SCD plasma (1.31 ± 1.74 ng/ml) was 30-fold higher than sCD40L found in normal plasma (0.04 ± 0.05 ng/ml, Figure 1). This value is several fold higher than in chronic lymphocytic leukemia[19] and acute coronary syndromes[18] where sCD40L is thought to contribute to the disease process. CD40 ligand levels in SCD plasma varied greatly among patients, ranging from normal values (*i.e.* 0.03 ng/ml) to values 150-fold higher (*i.e.* 6.0 ng/ml). sCD40L levels of SCD patients in crisis were further elevated by 79% when

compared to steady state (Figure 1), suggesting that higher levels of sCD40L correlate with the clinical status of SCD patients.

Platelet CD40L is decreased in SCD and further depleted during crises

Since more than 95% of circulating CD40L is contained within platelets[27] and released following platelet activation,[16] we examined the possibility that the chronically activated platelets believed to exist in SCD patients[28-30] might be the source of sCD40L in SCD plasma. Using the same ELISA assay, CD40L levels were measured from resting platelet lysates of SCD patients and normal individuals. We found that platelets from SCD patients contained less than half of the CD40L found in platelets from normal individuals (5.69 ± 4.81 ng/3x10^8 platelets versus 13.30 ± 6.77 ng/3x10^8 platelets, Figure 2A). This two-fold difference is more than enough to account for the elevated CD40L measured in the plasma of SCD patients.[31] Furthermore, the resting platelets of SCD patients in crises contained less than half of the CD40L than platelets of patients in the steady state (2.97 ± 2.81 ng/3x10^8 platelets versus 7.08 ± 5.26 ng/3x10^8 platelets, Figure 2B). These results provide evidence that platelets are a major source of elevated CD40L in SCD and suggest an association between decreased platelet CD40L, increased plasma CD40L and painful crises in SCD patients.

Tissue factor levels are elevated in SCD and correlate with elevated CD40L

Since CD40L mediates expression of the coagulation initiator TF,[23] we speculated that elevated CD40L may contribute to hypercoaguability in SCD plasma by upregulating TF production. Therefore, we compared the levels of TF in SCD and normal plasma (713.11

pg/ml ± 420.84 pg/ml vs. 6.34 pg/ml ± 12.34 pg/ml, Figure 3A). SCD plasma contained over 100-fold greater TF than the plasma from normal controls. Furthermore, elevations in TF correlated with elevations in sCD40L in matched SCD plasma samples at a level of R^2 = 0.600 (Figure 3B).

CD40:CD40L interaction augments SCD plasma-induced TF production by monocytes

Monocytes are known to respond to CD40L stimulation with increased TF production, thus promoting procoagulant activity.[32] We therefore asked whether SCD plasma increases TF production by monocytes, and if so, whether this increase is dependent upon the CD40:CD40L interaction. Lysates of monocytic THP-1 cells were assayed for TF following incubation with plasma from either SCD patients or normal volunteers. SCD plasma induced a significant increase in TF production relative to plasma from normal individuals or media alone. Optical density readings indicating relative TF expression were two-fold greater in lysates from SCD plasma-treated THP-1 cells than from THP-1 cells treated with normal plasma (Figure 4A). The CD40:CD40L interaction was not solely responsible for the elevation in TF production, but appeared to promote coagulation in some cases as pre-incubation of the THP-1 cells with a function blocking anti-CD40 antibody, prevented increased TF expression in 5 of 8 individual cases (Figure 4B).

SCD plasma-induced expression of VCAM-1 and ICAM-1 occurs via CD40:CD40L interaction

Red blood cells from SCD patients are known to induce endothelial cell expression of adhesion molecules.[33] We found that SCD plasma can also induce surface expression of endothelial VCAM-1 and ICAM-1. Relative to normal plasma, optical density readings were two-fold greater corresponding to surface expression of endothelial VCAM-1 and three-fold greater corresponding to surface expression of endothelial ICAM-1 as a result of SCD plasma treatment (Figure 5A). Similar to the results obtained with TF production, CD40 blockade significantly reduced SCD plasma-induced ICAM-1 expression by HUVEC (Figure 5B), suggesting that the CD40:CD40L interaction can contribute to the adhesive state of the endothelium by inducing ICAM-1 expression on these cells. The reduction in VCAM-1 expression was lowered by CD40 blockade, but was not statistically significant (Figure 5B).

CD40L in SCD plasma induces B cell proliferation

A B cell proliferation assay was used to further confirm the biological activity of soluble CD40L in SCD plasma. B cell proliferation was measured by ^{3}H-thymidine incorporation following the culture of Ramos B cells in either media alone, plasma from SCD patients or plasma from normal volunteers. We found that B cell incorporation of ^{3}H-thymidine was 31-fold greater in the presence of SCD plasma versus normal plasma (2.49×10^{-3} cpm ± 1.12×10^{-3} cpm vs. 0.079×10^{-3} cpm ± 0.028×10^{-3} cpm, Figure 6A). Since ligation of CD40 with anti-CD40 antibodies was found to activate Ramos B cells, the role of the CD40:CD40L interaction was investigated by immunoprecipitation of sCD40L from SCD plasma. Ramos B cells treated with sCD40L-cleared plasma exhibited significantly less proliferation than

control plasma, with an ~75% reduction in ^{3}H-thymidine uptake. Proliferation was not reduced by control IgG (Figure 6B), indicating that the increased proliferation of B cells by SCD plasma is CD40L-dependent and that the sCD40L circulating in SCD patients is biologically active.

Discussion

This study provides new insights into potential mechanisms contributing to inflammatory processes in SCD. CD40L has emerged as a potent mediator of inflammation, with elevated sCD40L levels being observed in a variety of diseases involving vascular inflammation.[17-19] We now find that sCD40L is elevated in SCD as well. Indeed, our findings indicate an average 30-fold elevation of sCD40L in SCD versus normal plasma, with corresponding decreases in the amount of CD40L stored in the platelets of SCD patients. The elevated sCD40L in SCD plasma positively correlates with increased TF and participates in the induction of TF and ICAM-1 expression via its interaction with CD40. Biological activity of sCD40L in SCD plasma is further confirmed by its induction of B cell proliferation. Together, these data identify sCD40L as potentially important for both inflammation and coagulation in SCD, and suggest a previously unrealized participation of platelets in SCD pathogenesis.

The magnitude of sCD40L elevation in SCD (~30-fold) can be compared to the increased levels of sCD40L found in other chronic inflammatory conditions such as SLE (>20-fold),[17] chronic lymphocytic leukemia (2.7-fold)[19] and unstable angina (3-fold).[18] In all of these latter conditions, CD40L is thought to contribute to the disease state. The 30-fold elevation

of sCD40L reported here places CD40L among the inflammatory cytokines potentially sharing a causative role in vascular occlusion in SCD.

In addition to platelets, WBC in SCD patients are also depleted of CD40L (data not shown), suggesting that both may contribute to the elevated sCD40L in SCD plasma. However, since platelets contain more than 95% of the circulating CD40L,[27] and these stores are reduced by approximately 57% in SCD (Figure 2), platelets are likely to be the major source of sCD40L in SCD plasma. Indeed, SCD patients average 5.7 ng of CD40L per $3x10^8$ platelets compared to 13.3 ng of CD40L found in normal platelets (Figure 2). Assuming a platelet count of $3x10^8$ platelets/ml of plasma, 7.6 ng of CD40L relocated from platelets to the plasma would correspond to nearly a 200-fold increase over normal circulating levels of 0.04 ng CD40L/ml, suggesting that the amount depleted from SCD platelets could more than account for the 30-fold elevation found in SCD plasma (Figure 1). Given that elevated sCD40L levels return to baseline within two hours of cardiopulmonary bypass,[31] and that the amount of sCD40L at sites of thrombosis would presumably be more concentrated, the potential exists for soluble CD40L to reach far greater levels than reported here. The further depletion of platelet CD40L during crises suggests a correlation to worsened clinical status of SCD patients and leads us to propose that platelets contribute to the chronic inflammation in SCD by releasing CD40L into the plasma.

Increased *in vivo* platelet activation in SCD results in increased CD40L exposure to the platelet surface[34] Once exposed, the cleavage of CD40L from platelets results in the release of sCD40L[16] to the plasma and a loss of platelet CD40L. Therefore, SCD platelets have

more surface-exposed CD40L,[34] but less total CD40L stored, as measured here in platelet lysates (Figure 2).

CD40L may link chronic inflammation and hypercoagulation in SCD. We confirm here that TF, a major initiator of the coagulation cascade, is abnormally elevated in SCD plasma (Figure 3A).[24] Furthermore, elevated TF in SCD plasma correlates to increased CD40L (Figure 3B). Since monocyte TF production can be induced by SCD plasma (Figure 4) and reduced by CD40 blockage, our results suggest that the CD40:CD40L contributes to hypercoagulation in SCD, particularly when taken in context with other studies showing that CD40L upregulates TF production.[23,35,36] Platelets may therefore contribute to the hypercoagulation in SCD via CD40L exposure and release.

Notably, our study indicates that plasma from SCD patients is itself inflammatory. Plasma from SCD patients was sufficient to increase endothelial ICAM-1 expression 3-fold (Figure 5A), and Ramos B cell proliferation 31-fold (Figure 6A). Therefore, therapeutic approaches targeted to inflammatory plasma components may also be beneficial to SCD patients.

Recent evidence that CD40L provides a novel mechanism of platelet activation[37] suggests a potential positive feedback loop whereby CD40L participation in SCD may be perpetuated. CD40 ligand is thought to cause α– and dense-granule release, potentially maintaining the activation profile already characterized by SCD platelet studies.[30,34,39] CD40L-induced P-selectin exposure[38] may lead to further procoagulant activity[40] and strongly suggests that targeting CD40L release or activity may be therapeutically beneficial to SCD patients.

Inhibitors of platelet GPIIb-IIIa have shown promising results in acute coronary syndromes,[41,42] and anti-CD40L treatment reportedly improves the clinical profiles of SLE patients.[43] SCD patients may be candidates for either of these therapies. Future studies will be necessary to clarify the relative importance of CD40L in context with the other inflammatory mediators in SCD plasma, as well as to determine whether the elevated sCD40L levels in SCD are as predictive of inflammatory and thrombotic activity in SCD as they are in acute coronary syndromes.

Figure 1

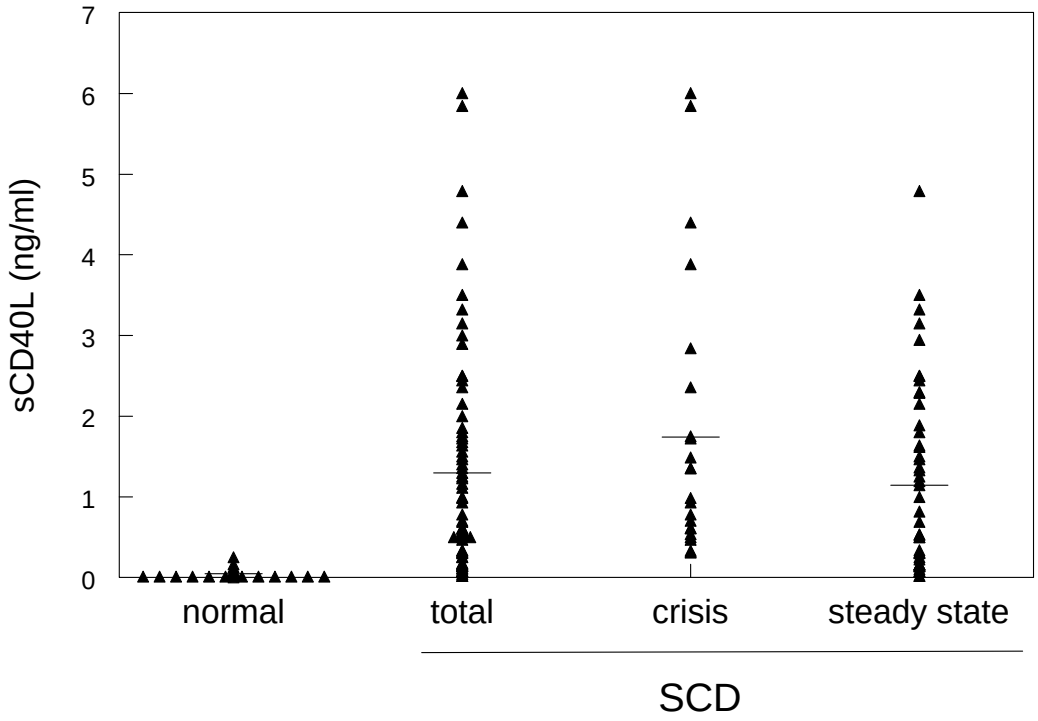

Figure 1. Soluble CD40L is elevated in SCD plasma. Levels of sCD40L were determined to be 1.18 ± 1.39 ng/ml in plasma samples from 49 sickle cell (SCD) patients compared to 0.04 ± 0.07 ng/ml in plasma from 16 normal volunteers by ELISA as extrapolated from a standard curve. Total SCD data are significantly different from normal controls (p-value < 0.0001). Crisis patients have even higher sCD40L levels at 1.89 ± 1.59 ng CD40L/ml of plasma from 10 SCD patients in pain crises compared to 1.05 ± 1.33 ng CD40L/ml of plasma from 37 steady state SCD patients (p-value = 0.065).

Figure 2A

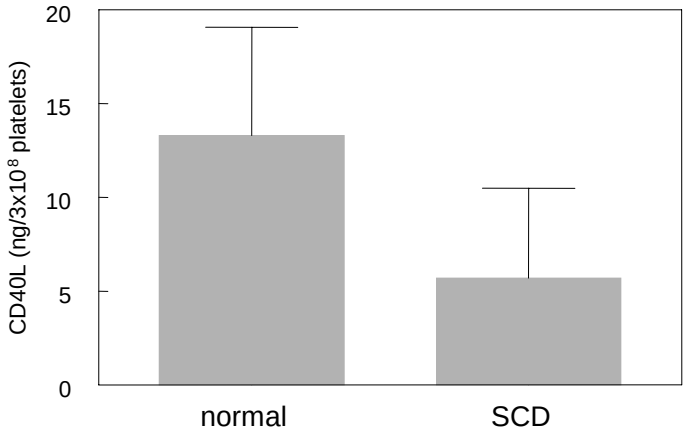

Figure 2B

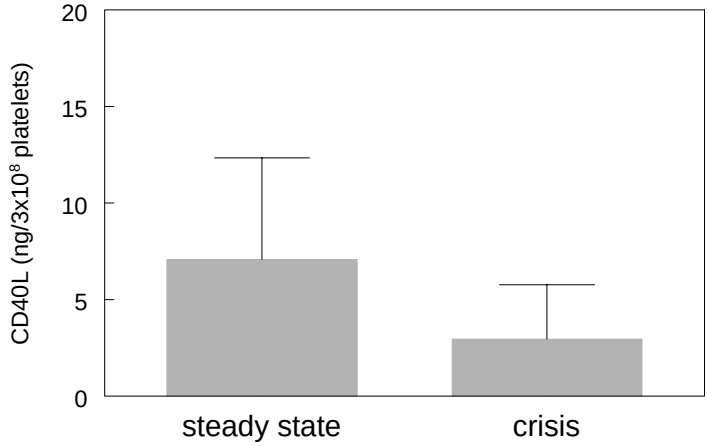

Figure 2. Platelets from SCD patients have lower CD40L levels than normal controls and are further depleted during crises. (A) Platelet lysates from 21 SCD patients contained 5.69 ± 4.81 ng CD40L/3 x 10^8 platelets, or less than half the 13.30 ± 6.77 ng CD40L/3 x 10^8 platelets found in 4 normal samples. Data reflect measurement by ELISA and are significantly different (p-value = 0.029). (B) Platelet lysates from 13 steady state patients contained 7.08 ± 5.26 ng CD40L/3 x 10^8 platelets, or more than two-fold greater than the average 2.97 ± 2.81 ng CD40L/3 x 10^8 platelets found in platelets from 8 SCD patients in crisis. Data reflect measurement by ELISA and are significantly different (p-value = 0.042).

Figure 3A

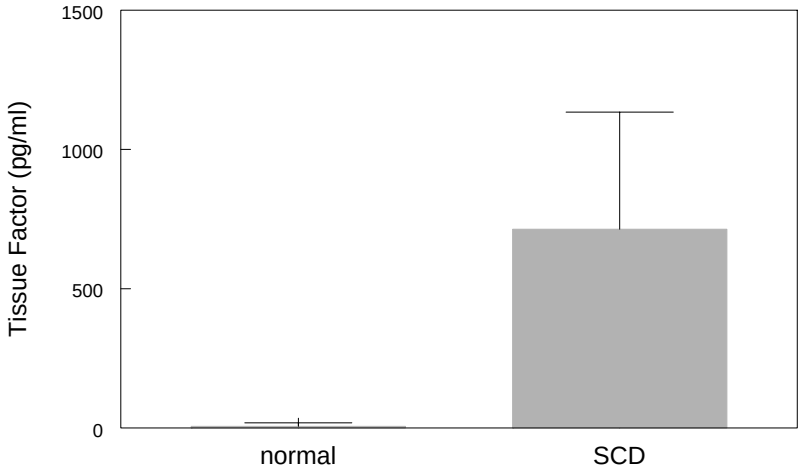

Figure 3B

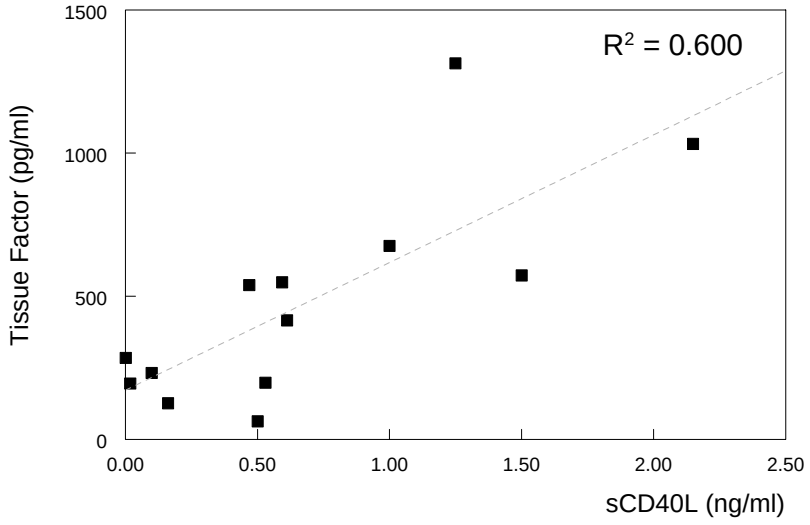

Figure 3. Tissue factor is elevated in SCD plasma and correlates with higher CD40L levels. (A) The coagulation cascade initiator TF was found to be elevated over 100-fold in 18 SCD patients compared to normal donors (713.11 pg/ml ± 420.84 pg/ml vs. 6.34 pg/ml ± 12.34 pg/ml). Data shown represent tissue factor levels as measured by ELISA and are significantly different (p-value = 0.0395). (B) Levels of TF in SCD plasma samples were plotted against corresponding sCD40L levels measured in the same samples. R2 = 0.600 for sCD40L levels < 2.5 ng/ml.

Figure 4A

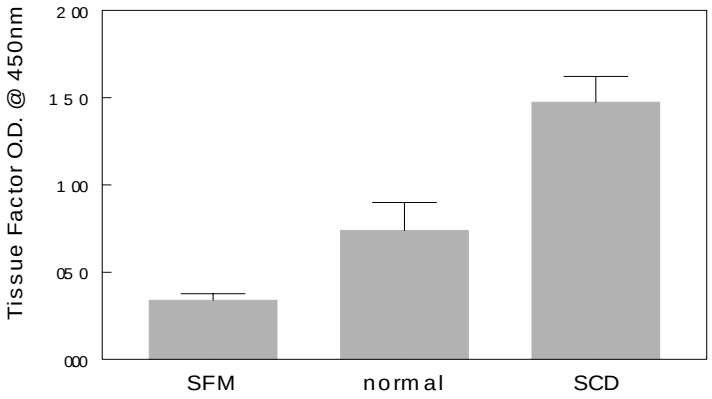

Figure 4B

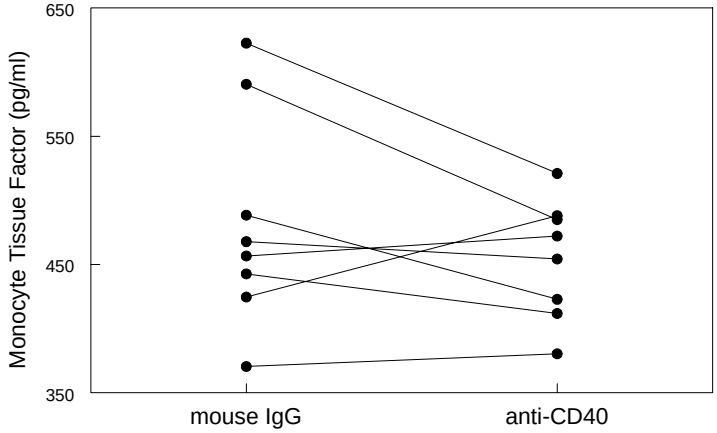

Figure 4. SCD plasma-induced monocytic TF is reduced by an anti-CD40 monoclonal antibody. (A) Monocytes exposed to plasma from SCD patients produced greater TF than monocytes exposed to plasma from normal volunteers. Optical density readings of TF in THP-1 lystates are 2X greater following treatment with SCD plasma. (B) In 5 of 8 SCD patient samples, an anti-CD40 monoclonal antibody decreased TF production by THP-1 cells relative to an isotype-matched control antibody.

Figure 5

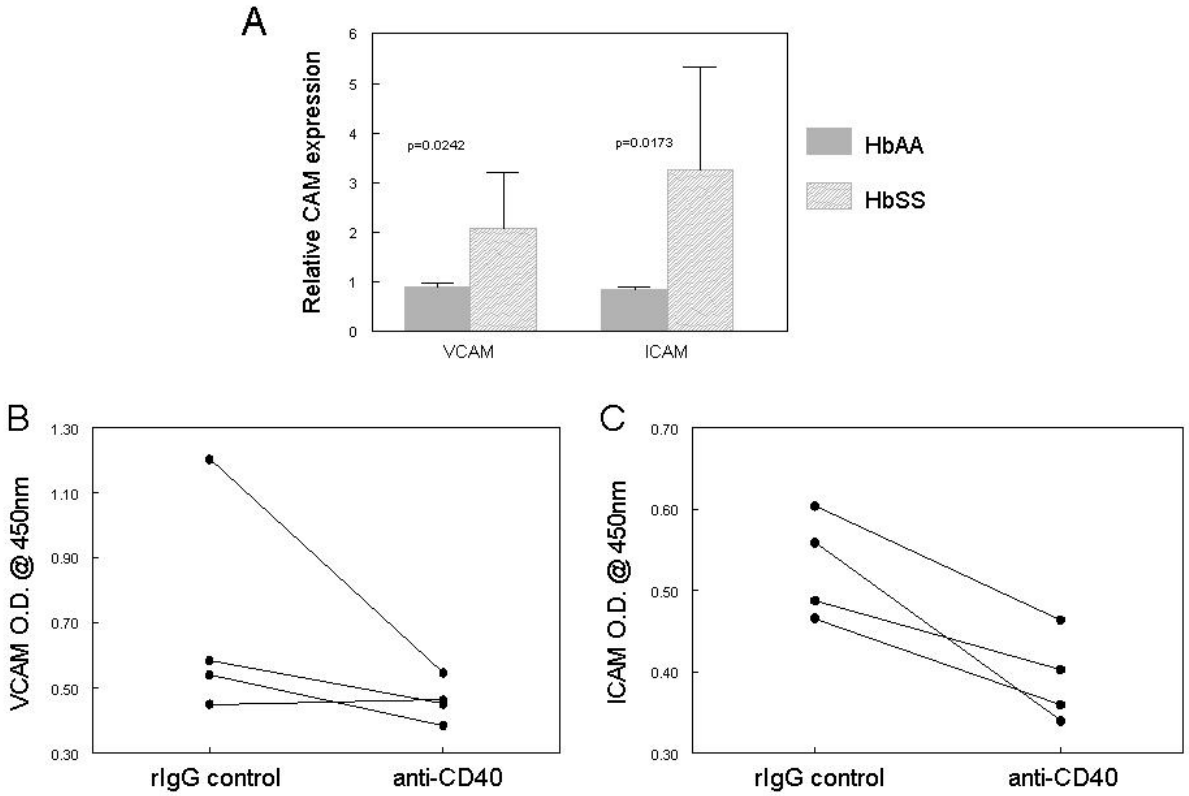

Figure 5. Blockage of CD40 on HUVEC inhibits SCD plasma-mediated increases in VCAM-1 and ICAM-1 expression. (A) Optical density readings corresponding to HUVEC surface expression of adhesion molecules were increased two-fold for VCAM-1 and three-fold for ICAM-1 (p-value = 0.0242) following treatment with SCD plasma from 8 patients compared to plasma from 3 normal individuals. (B) ICAM-1 expression could be significantly reduced by pretreatment of HUVEC with an anti-CD40 polyclonal antibody (n = 4, p-value = 0.0475). The reduction in VCAM-1 expression was not statistically significant. Non-specific rabbit immunoglobulin had no effect.

Figure 6A

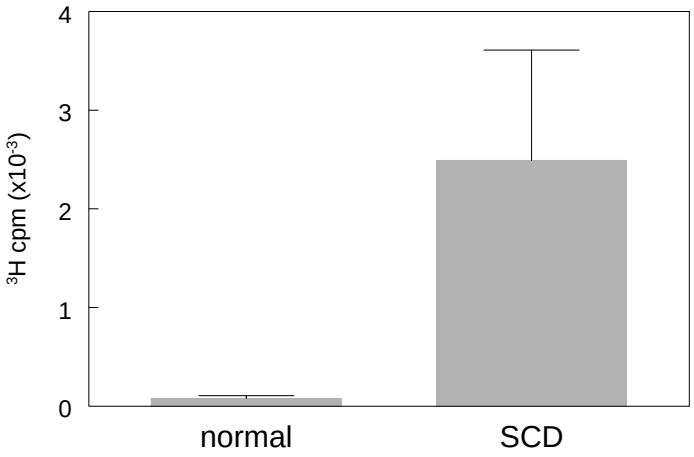

Figure 6B

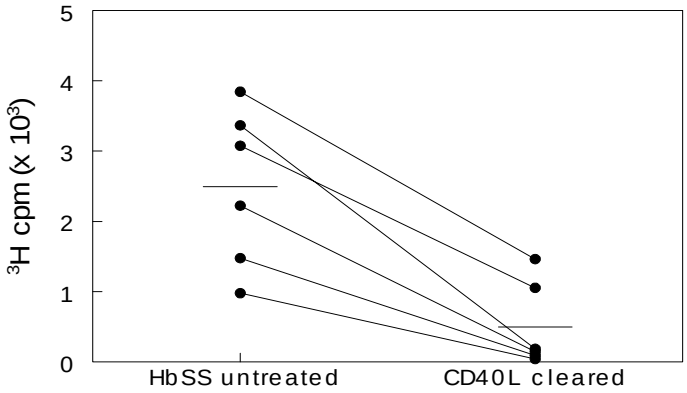

Figure 6. B cell proliferation increases 35-fold in SCD vs. normal plasma and is inhibited by CD40L depletion from plasma. (A) Ramos B cells were cultured in RPMI-1640 + 10% human plasma from either SCD patients or normal volunteers. Uptake of 3H-thymidine averaged 0.079 x 10-3 cpm ± 0.028 x 10-3 cpm in the presence of plasma from 3 normal volunteers and ~31-fold higher (2.49 x 10-3 cpm ± 1.12 x 10-3 cpm) in the presence of SCD plasma from 6 patients. (B) Depletion of sCD40L by treating SCD plasma with beads plus anti-CD40L polyclonal antibody significantly decreased 3H- thymidine uptake by an average of 75% to 0.50 x 10-3 cpm ± 0.61 x 10-3 cpm, versus a non-specific rabbit IgG control antibody, which did not inhibit SCD plasma-induced B cell proliferation (p-value = 0.002).

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CHAPTER III.

Specific Aim 2. Determine if CD40L contributes to SCD pathology *in vivo*.

Rationale

CD40 ligand (CD40L) is a TNF family member[1] implicated in the pathogenesis of SCD.[2] Platelet-released CD40L may participate in SCD severity via numerous mechanisms. CD40L may be partially responsible for high leukocyte counts[3] that are predictive of SCD severity[4] and early death[5] because of the role CD40L plays in inducing leukocyte proliferation.[6,7] Endothelial adhesion molecule expression may be upregulated by CD40L,[8] potentially leading to increased sickle RBC adhesion to the endothelium.[9] Sickle cell adhesion may also follow extracellular matrix exposure[10] caused by a CD40L-mediated increase in MMP production.[11] Combined with the ability of CD40L to promote coagulation,[12] these findings suggest a role for CD40L in SCD vaso-occlusion.

Sickle cell disease is known to be a chronic inflammatory condition[13] where multiple inflammatory mediators are upregulated.[14-16] Elevations in inflammatory cytokines and acute phase proteins[17] are implicated in disease severity[18] and pathology[19]. Cytokines may play a role in SCD by inducing endothelial adhesiveness[20,21], activating platelets and neutrophils[15], regulating hematopoiesis[22], and inhibiting immune functions.[23] All of the aforementioned factors likely contribute to the exacerbation SCD severity. Since the manifestations of SCD

are likely the combined result of numerous factors, the relative role played by CD40L in this inflammatory milieu remains obscure. Therefore, we sought to determine the relative importance of CD40L to SCD pathology *in vivo* using a mouse model of SCD.[24] To accomplish this, sCD40L was measured in platelet-free plasma from SCD mice. Mice mimicking SCD were also treated with an anti-CD40L monoclonal antibody shown to effectively block CD40L activity in a murine model of atherosclerosis.[25] Accumulated organ damage in anti-CD40L treated mice was then compared to mice receiving non-specific immunoglobulin control. In addition, the relative importance of CD40L recognition by the endothelium will be determined by imposing the SCD phenotype onto mice lacking expression of the known possible CD40L receptors, CD40 and β3 integrin.[26]

Materials and Methods

CD40L ELISA

Blood was drawn by cardiac puncture from wild-type, sickle and heterozygote mice into ACD in the laboratory of Dr. Paul Frenette (Mt. Sinai Medical Center, New York, NY). Blood cells were removed by a 200*g* centrifugation for 15 min, followed by resting the resultant platelet-rich plasma at 37°C for 15 min. Prostaglandin E_1 was added to maintain platelet quiescence before a second centrifugation of 750*g* for 20 min to remove platelets. Platelets lysed in 1%NP-40 and platelet-free plasma were stored at -80°C and shipped on dry ice where samples were used in a sandwich ELISA. Briefly, CD40L was captured by an anti-mouse CD40L monoclonal antibody-coated microtiter wells. CD40L was then sandwiched by an anti-CD40L polyclonal antibody, that was in turn detected by a horseradish peroxidase

(HRP)-linked anti-rabbit secondary antibody. A tetramethyl benzidine developing solution (Sigma Chemicals) was applied as the HRP substrate and the color reaction was stopped by adding 2N H_2SO_4. Color was then read at 450nm by a microplate reader and compared to a standard curve of known concentrations of recombinant mouse CD40L.

Bone Marrow Transplantation

Eight-week old wild-type C57BL/6, CD40-/- or β3-/- females were given two doses of total body irradiation, 700 Rad and 500 Rad given 4 hours apart, in a cesium-137 irradiator (AECL Gammacell 40). Lethally irradiated mice were then rescued with an injection of $3x10^6$ bone marrow cells collected from the hind limbs of a genetically bred SCD mouse[24] donor. In this way, bone marrow transplantation reliably confers the hematopoietic phenotype of donor mice unto the genetic background of irradiated recipients.[27] In addition to standard chow, mice were maintained on acidified neomycin water (1g/L, pH 2.0) for 2-3 weeks following transplant. Chimerism was verified by hemoglobin electrophoresis 6-8 weeks following the transplant. All mice were housed in a barrier facility maintained by the UNC Department of Laboratory and Animal Medicine (DLAM). Protocols were reviewed and approved by the UNC Institutional Animal Care and Use Committee (IACUC).

Anti-CD40L treament

Bone marrow transplanted (BMT) SCD mice were treated with a monoclonal antibody, clone MR1, known to inhibit CD40L activity *in vivo*.[25] Mice were injected intraperitoneally with 250 μg of low-endotoxin, azide-free anti-CD40L antibody (Taconic) twice per week for 6

weeks. Non-specific, isotype-matched control antibody was also administered to BMT SCD mice as control. Following treatment, blood was drawn by cardiac puncture.

Organ collection and histologic preparations

Mice were weighed, then placed in a CO_2 inhalation chamber until motionless. Mouse organs were preserved by cardiac infusion of PBS followed by freshly prepared 4% paraformaldehyde, pH 7.2. The fixation process was continued by incubation of whole mice in 4% paraformaldehyde for 7 days. Mice were then switched to a solution of 70% ethanol until organs were collected for histologic preparation. Lung, liver, kidney and spleen were embedded in paraffin, sectioned and mounted on slides. All organs were stained with hematoxylin & eosin (H&E), gomori iron and the fibrin-indicator phosphotungstic acid-hematoxylin (PTAH) stains. Paraffin preparation, tissue sectioning and histological staining were performed by the DLAM histopathology core facility.

Results

Soluble CD40L may be elevated in SCD mouse plasma and depleted from mouse platelets

To determine if SCD mice exhibit elevated plasma CD40L levels as seen in humans, an ELISA was developed to measure CD40L in mouse platelet-free plasma and platelet lysates. Results from samples collected in the laboratory of Paul Frenette (Mt. Sinai, New York, NY) demonstrated that plasma CD40L is 1.3X greater in an SCD mouse (SS) compared to wild-type (WT), while platelet CD40L is measurably less in the SCD mouse compared to wild-type (Figure 7). The heterozygote mouse (SA) expressing one sickle allele also exhibited

elevated plasma CD40L, although platelets from this mouse did not contain less CD40L (Figure 7).

Splenic abnormalities in BMT SCD mice are reduced by anti-CD40L treatment, but not by the absence of potential CD40L receptors from the endothelia

To determine the relative contribution of CD40L to SCD *in vivo*, SCD mice were generated through bone marrow transplantation, then treated with the anti-CD40L antibody, clone MR1, shown to block CD40L activity in a mouse model of atherosclerosis.[25] The SCD phenotype was also conveyed to CD40-/- and β3-/- mice to determine the importance of endothelial recognition of CD40L. Spleens collected from anti-CD40L treated mice did not exhibit the dramatic 7-fold enlargement that is characteristic of SCD mice. Anti-CD40L treated mouse spleens, but not IgG control treated, were comparable to normal in percentage of mouse body weight (Figure 8A). Conversely, the absence of CD40 and β3 integrin from the endothelia did not result in decreased spleen size (Figure 8A). Bone marrow transplantation alone did not increase spleen size, as WT BMT controls did not exhibit splenic enlargement (Figure 8A).

Upon microscopic examination of spleens, the characteristic disruption of splenic architecture that is typical in SCD mice was readily observed in BMT mice. Red-white pulp nodular organization was apparent in mice treated with anti-CD40L, but not control IgG (Figure 8B). The lack of endothelial CD40 nor β3 integrin was protective against the abnormal splenic architecture induced by the SCD BMT (Figure 8B).

Lung pathology in BMT SCD mice is abrogated by both anti-CD40L treatment and the absence of CD40

BMT mice were treated with anti-CD40L antibody, an isotype-matched control IgG, or left untreated for 8-10 weeks following the introduction of SS bone marrow. Histologic preparations of perfusion-fixed lung tissue were prepared with H&E, iron and fibrin stains. Lungs from anti-CD40L treated mice, compared to lungs from control IgG-treated mice, had demonstrably less damage as evidenced by the amount of iron and fibrin deposits (Figure 9). The lack of CD40 was also protective against sickle-induced lung pathology in the CD40-/- chimeric mice (Figure 9), with lungs virtually free of iron and fibrin deposits. Conversely, the absence of the β3 integrin did not affect accumulated damage to the lungs of the β3-/- mice following the sickle BMT procedure (Figure 9).

Anti-CD40L treatment reduces liver and kidney pathology in BMT SCD mice

Liver and kidney tissue sections isolated from anti-CD40L treated, BMT SCD mice were also prepared, staining for iron and fibrin deposits. Liver sections from the anti-CD40L were less positive for both iron and fibrin stains than liver sections from mice treated with a non-specific, isotype-matched control antibody (Figure 10). Kidney sections from the anti-CD40L treated mice contained demonstrably less iron deposits than the IgG control treated mice, however fibrin deposits appeared similar for both treatment groups (Figure 10).

Non-hematopoietic sources of CD40 and β3 integrin are not required for SCD-induced liver and kidney damage.

Perfusion-fixed liver and kidneys were isolated from chimeric SCD mice expressing the sickle phenotype against either a CD40-/- or β3-/- background. When histologic sections of liver and kidney tissue were examined, the absence of CD40 nor β3 integrin from the non-hematopoietic cells appeared to protect the BMT mice from SCD-induced iron and fibrin deposits (Figure 11).

Discussion

CD40L is a potent inflammatory mediator[1] known to induce proliferation of some leukocytes.[6] Upon recognition by its receptor CD40, CD40L also increases endothelial expression of cell adhesion molecules[1,8] and promotes coagulation through upregulated tissue factor production.[28,29] The elevated WBC counts[5,13], endothelial adhesivity[30,31] and hypercogulation[32] that exacerbate the clinical course of SCD, may be partially explained by the activity of CD40L.[2] However, multiple inflammatory cytokines are likely involved in the clinical manifestations of SCD.[15] In this study, we sought to determine the relative importance of CD40L to SCD pathology *in vivo*.

Results presented here infer that sCD40L may be elevated in the plasma of SCD mice (Figure 7) just as it is in the plasma of human SCD patients.[2] As these data only represent a single mouse of each genotype, firm conclusions can not be drawn. However, the higher level of sCD40L found in plasma from a SS homozygote corroborates the fact that the

phenotype of the Berkeley SCD mice[24] strongly recapitulates human SCD.[33] The potential elevation of sCD40L in mouse SCD plasma also lends credence to the relative importance of this particular characteristic.

When the activity of CD40L was blocked in the SCD mice, we found that organ pathology was noticeably diminished. Namely, the characteristic enlargement of the spleen that occurs in SCD was not evident following anti-CD40L treatment (Figure 8A). In fact, spleens of anti-CD40L treated mice were normal in size rather than the gross 7-fold splenic enlargement of the IgG control-treated mice (Figure 8A). Furthermore, well-defined nodules indicative of normal red-white pulp splenic architecture was evident in anti-CD40L treated mouse spleens, but not in the spleens of mice treated with an isotype-matched control antibody (Figure 8B). Larger, more defined areas of white pulp seen in the spleens of the anti-CD40L treated mice are seemingly the antithesis of expected results since CD40L is known to mediate proliferation of leukocytes found in white pulp.[1,6] However, it is important to note that the sections shown here are indicative of architectural organization only and do not allow for quantitative comparisons of particular cell types to be made. Regardless, this study becomes the first to demonstrate that CD40L participates in SCD-induced splenic enlargement and the disruption of splenic architecture.

The data shown here go on to demonstrate that the CD40:CD40L dyad plays a prominent role in the development of lung pathology in SCD. Lung tissue from BMT SCD mice were virtually free of iron and fibrin deposits when either CD40L was blocked by anti-CD40L treatment or when CD40 was absent from non-hematopoietic cells (Figure 9). As the

absence of non-hematopoietic CD40 did not affect SCD-induced pathology of other organs studied (Figure 11), these results highlight differential and organ-specific mechanisms by which CD40L participates in the pathology of SCD. Since anti-CD40L treatment demonstrably reduced SCD-induced organ pathology (Figures 8 and 10) in tissues where the absence of non-hematopoietic CD40 and endothelial β3 integrin had no effect (Figures 8 and 11), these results suggest that: 1) CD40L may interact with an unknown receptor; 2) Endothelial expression, rather than recognition, of CD40L may be an important mechanism of organ damage induced by SCD; or 3) Hematopoietic sources of CD40 and CD40L participate in SCD-induced organ damage. Additional studies aimed at dissecting out the specific mechanisms by which CD40L participates in SCD pathology are warranted.

Figure 7

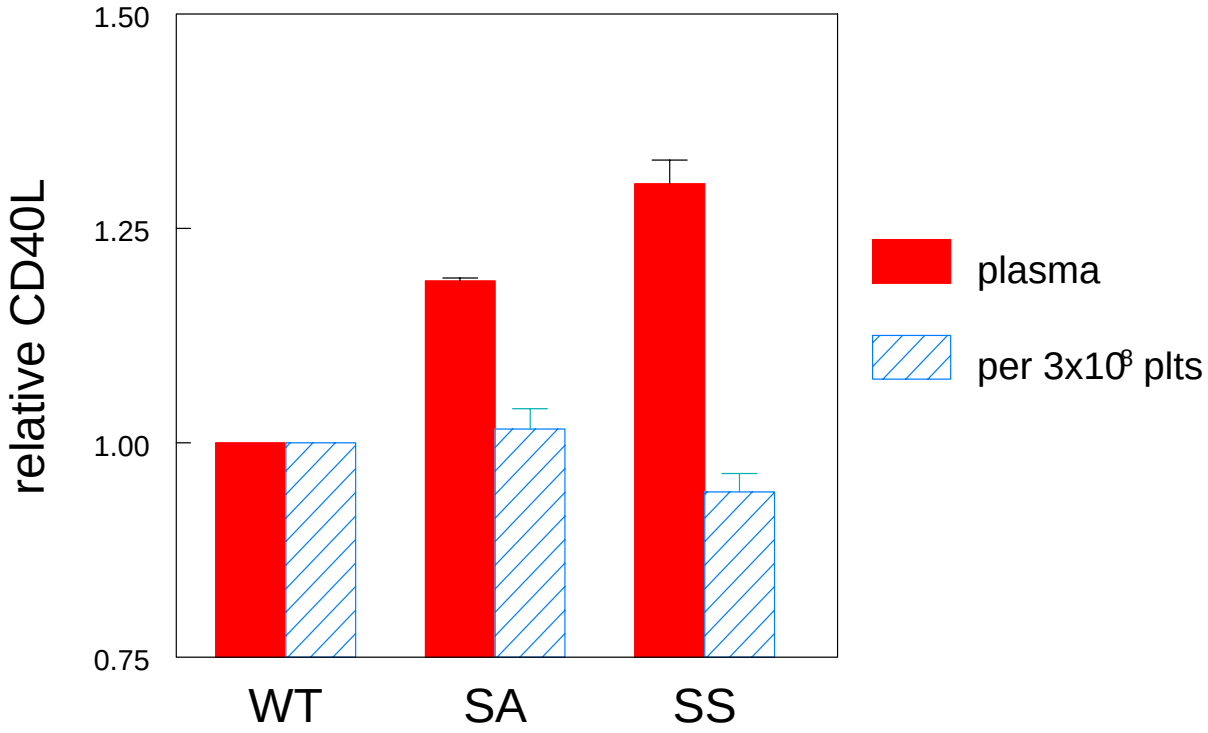

Figure 7. CD40L may be elevated in mouse plasma and depleted in mouse platelets. CD40L was determined to be 0.265 ± 0.001 ng/ml in the plasma of a wild-type C57BL6 mouse (WT) and 0.317 ± 0.036 ng/3x10^8 platelets. A heterozygote expressing one human sickle allele (SA) had plasma CD40L of 0.315 ± 0.001 ng/ml and 0.322 ± 0.008 ng/3x10^8 platelets. The homozygote SCD mouse (SS) had plasma CD40L of 0.345 ± 0.027 ng/ml and platelet CD40L of 0.299 ± 0.007 ng/3x10^8 platelets. Results shown are normalized to wild-type measurements and reflect 3 iterations of 1 sample from each phenotype.

Figure 8

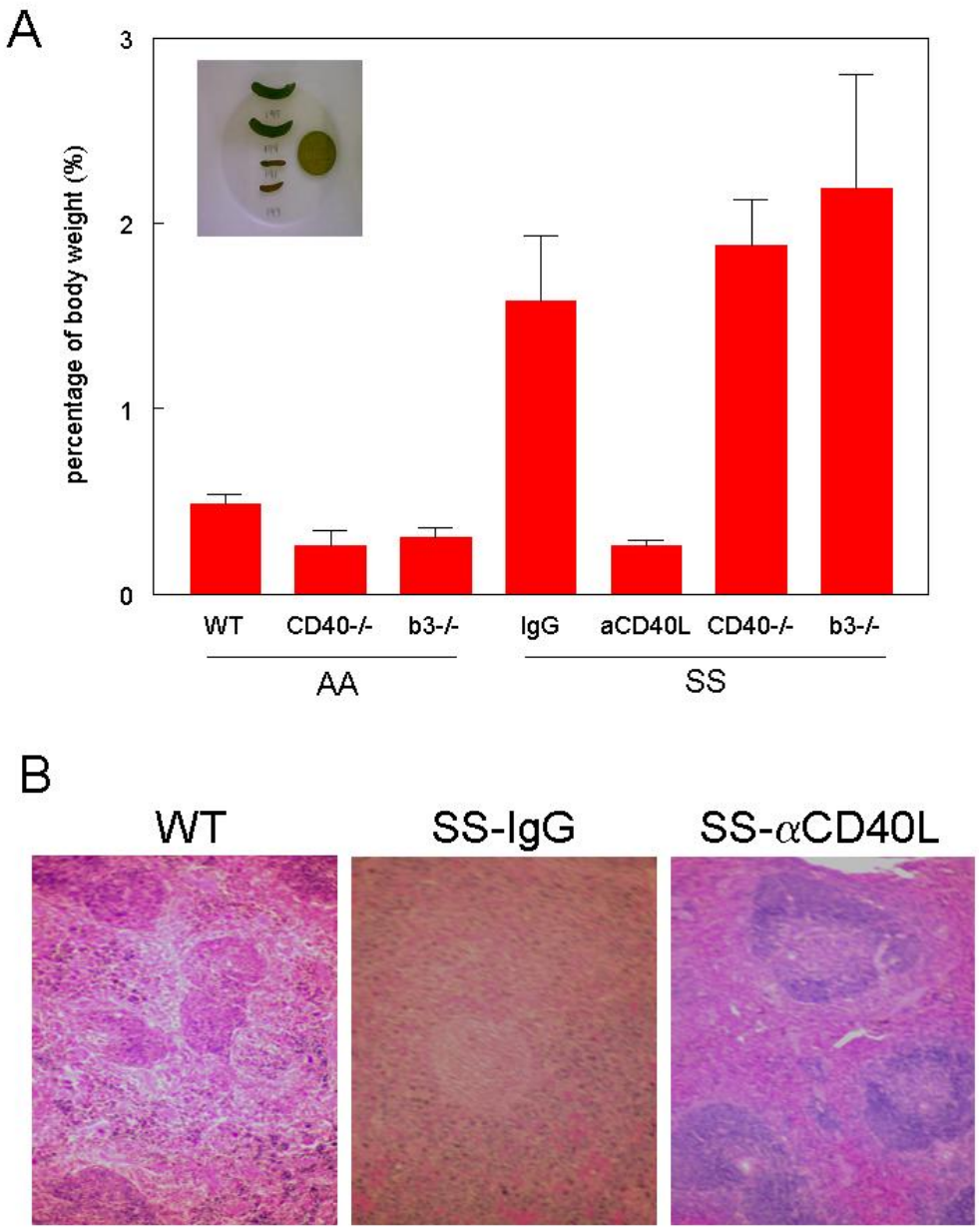

Figure 8. SCD-induced splenic abnormalities are reduced by anti-CD40L treatment, but not by the absence of endothelial CD40 and β3 integrin in BMT SCD mice. (A) The spleens of SS BMT mice (n=4) averaged 1.585% of total body weight compared to just 0.485% in AA BMT mice (n=2). In CD40-/- mice, spleens were 1.88% and 0.263% in SS (n=3) and AA (n=3) BMT mice, respectively. Spleens from β3-/- mice averaged 2.19% in SS BMT mice (n=2) and 0.305% in AA BMT mice (n=2). Following treatment with an anti-CD40L antibody, spleens of SS BMT mice averaged 0.285% of total body weight. (B) Photographs of 10X magnification of spleen sections stained with H&E demonstrate that anti-CD40L treated mouse spleens have the nodular red-white pulp architecture that is lost in SCD. IgG control treatment had no effect on spleen size or architecture.

Figure 9

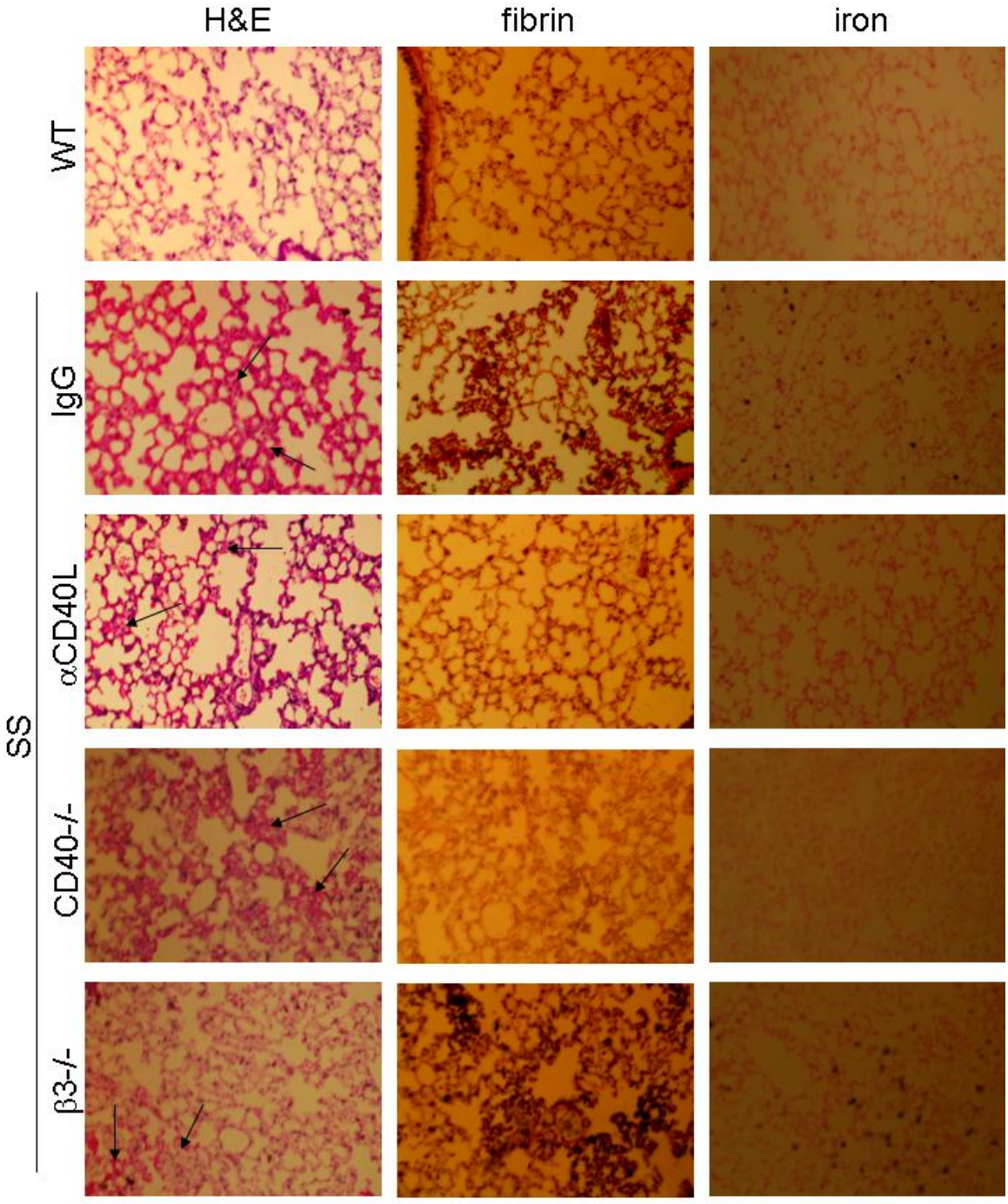

Figure 9. **Lung pathology in BMT SCD mice is abrogated by both anti-CD40L treatment and the absence of CD40.** RBC congestion (black arrows) is evident in H&E stained lung sections of SS BMT mice, but not in AA BMT controls. Fibrin and iron deposits are noticeably decreased by αCD40L treatment as well as the absence of non-hematopoietic CD40. IgG control treatment and the lack of non-hematopoietic β3 integrin had no effect on SCD-induced fibrin and iron deposits. Data shown represent photographs of 20x magnification of lung sections.

Figure 10

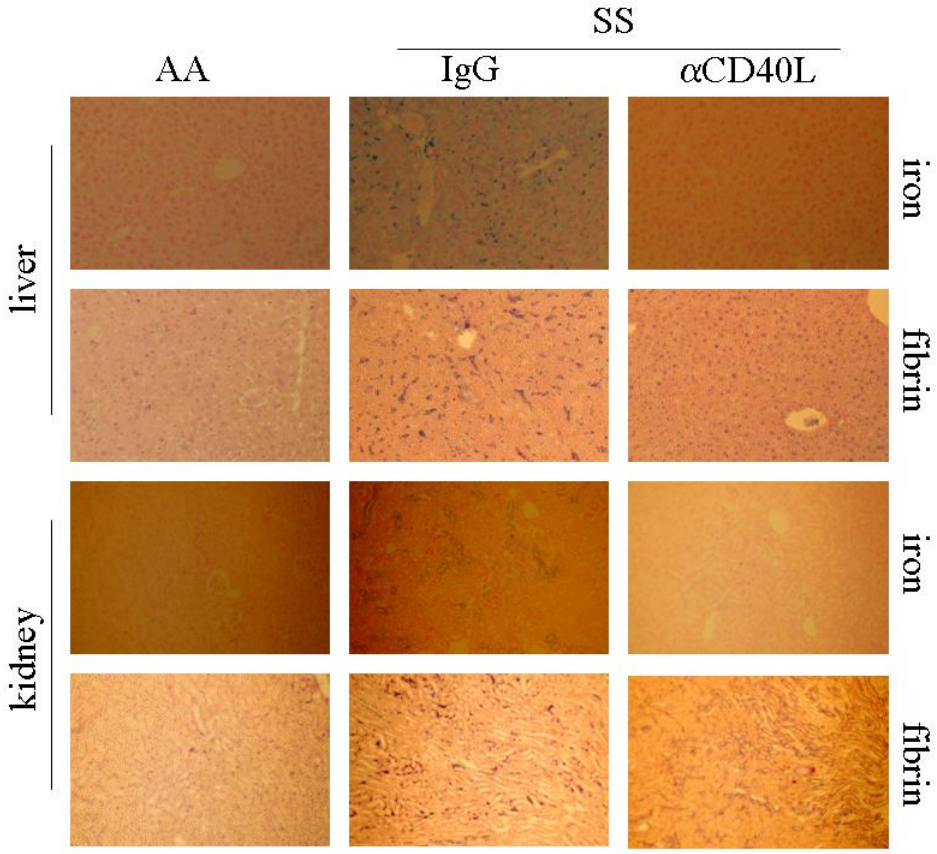

Figure 10. Anti-CD40L treatment decreases liver and kidney pathology in BMT SCD mice. Liver and kidney sections from SS BMT mice contain iron and fibrin deposits not found in comparable sections from AA BMT control mice. αCD40L treatment reduced iron deposits in both liver and kidney sections. Fibrin deposits were visibly reduced in liver, but this reduction was not readily apparent in kidneys. Isotype-matched control IgG treatment did not reduce SCD-induced iron and fibrin deposits to liver and kidney in BMT mice. Data shown represent photographs of 20X magnification of liver sections and 10X magnification of kidney sections.

Figure 11

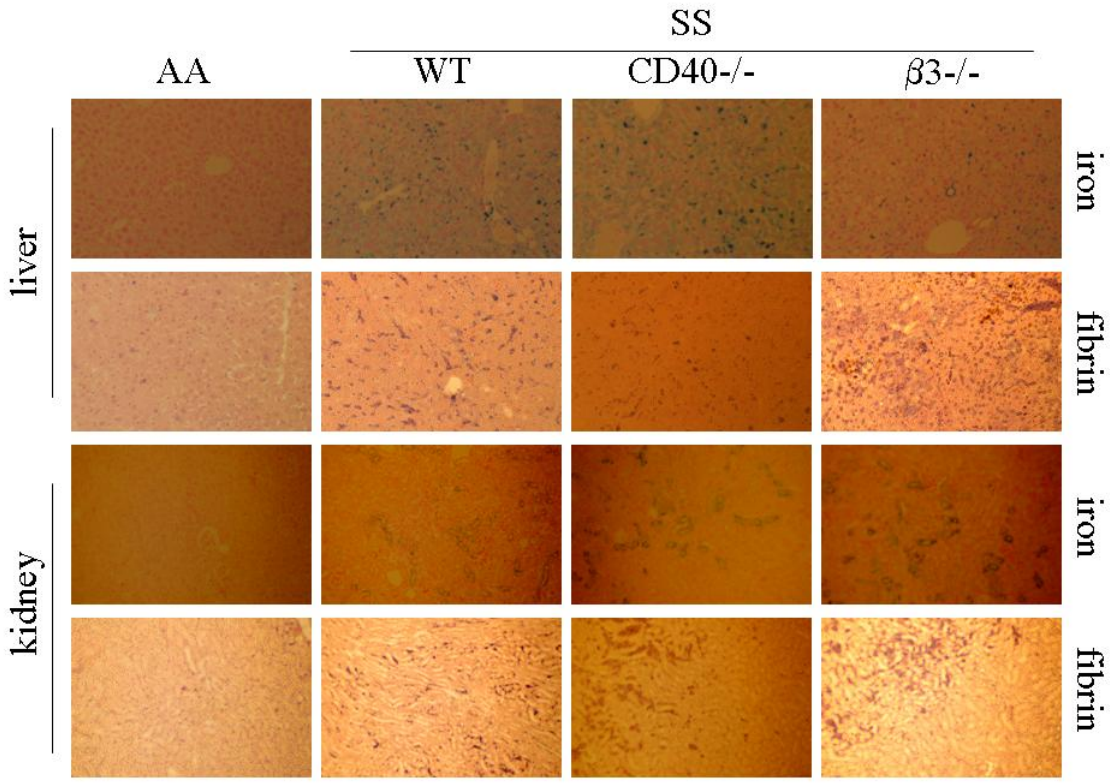

Figure 11. Non-hematopoietic sources of CD40 and β3 integrin are not required for SCD-induced liver and kidney damage. Liver and kidney sections from SS BMT mice contain iron and fibrin deposits not found in comparable sections from AA BMT control mice. These deposits of iron and fibrin were evident regardless if the SS phenotype was transplanted onto a WT, CD40-/- or β3-/- background. Data shown represent photographs of 20X magnification of liver sections and 10X magnification of kidney sections.

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CHAPTER IV.

Specific Aim 3. Determine the role of platelet activation in SCD.

Rationale

Sickle cell disease (SCD) is a complex syndrome characterized by a hypercoagulable state of multifactorial etiology[1]. A quagmire of vaso-occlusive RBC phenomena[2] is compounded by ischemia-reperfusion injury,[3] elevated leukocyte counts, abnormal activation of granulocytes and monocytes,[4,5] and elevated levels of multiple inflammatory mediators.[2] Platelets are also thought to contribute to SCD via P-selectin,[6-8] endothelial activation[9] and aggregate formation.[10] Platelet-released factors[11] increase RBC adhesivity,[12,13] coagulation[14] and vasoconstriction.[15,16]

Furthermore, platelets expose and release the inflammatory mediator CD40L,[17] capable of mediating a broad variety of immune and inflammatory responses.[18] As SCD is characterized by elevated leukocyte counts,[19] circulating endothelial cells[20] and a hypercoaguable state,[21] platelet CD40L may play a similar role in SCD as it does in other vascular diseases.[22,23] Our findings that the elevation of biologically active soluble CD40L in plasma correlates to the SCD clinical state[24] further suggest that understanding platelet activation and subsequent CD40L release may be essential for understanding and alleviating symptoms of SCD.

The integrin αIIbβ3 is critical for platelet aggregation, adhesion, granule secretion, and platelet-induced procoagulant activity.[25,26] Blockade of αIIbβ3 inhibits thrombotic vessel occlusion,[27] the prothrombin activation that leads to coagulation,[28] as well as platelet release of inflammatory CD40L.[29] The antagonism of αIIbβ3 may therefore be beneficial to SCD patients as it may reduce many of the factors contributing to the adhesive, pro-inflammatory, and hypercoaguable state of the SCD vasculature.

Eptifibatide, or Integrilin®, is an αIIbβ3 antagonist shown to be safe and effective in treating acute coronary syndromes (ACS).[30,31] Blockade of αIIbβ3 via eptifibatide also effectively decreases the release of platelet CD40L,[29] suggesting possible therapeutic benefit to SCD patients. Therefore, we asked if eptifibatide could safely lower CD40L levels when administered to patients with SCD. We also assessed changes in the inflammatory profile of eptifibatide-treated SCD patients and determined if comparable pharmacodynamics observed in the treatment of ACS could be achieved within the complex hemodynamics of SCD.

Materials and Methods

Study Participants

Approval for this study was obtained from the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina-Chapel Hill with informed consent provided according to the Declaration of Helsinki. Study participants were eligible if they met the following criteria: 1) adults (age 18 to 50 years) with a confirmed diagnosis of homozygous SCD (Hb SS); 2) had no history of acute vaso-occlusive events requiring

hospitalization over the preceding 6 weeks; 3) had clinically acceptable values for hematology, chemistry, urinalysis and electrocardiogram for a patient with SCD; 4) had a negative pregnancy test, if female; 5) had a clinically acceptable physical examination; 6) had no evidence of infiltrates on a chest x-ray; and 7) weighed ≤ 100 kg. Patients were excluded from participating in the study if: 1) they were pregnant or breastfeeding; 2) had laboratory values that indicated major organ dysfunction (e.g. serum creatinine > 2.0, AST and/or ALT > 3 times normal); 3) had received a red blood cell transfusion within the previous 3 months; 4) had a history of clinically significant active cardiovascular, neurologic, endocrine, hepatic or renal disorder; 5) uncontrolled hypertension; 6) previous hemorrhagic stroke; 7) had a history of recent illicit drug or alcohol abuse; 8) had been exposed to any investigational drug within the preceding 6 weeks; 9) they were on chronic anticoagulation therapy; and 10) they were on aspirin, non-steroidal anti-inflammatory drugs or other anti-platelet medications.

Patient Characteristics

Four subjects were enrolled onto this open-label Phase I study. All of the subjects were studied in their non-crisis, steady states. All study subjects were male and had homozygous SCD (Hb SS). The median age was 31.5 years (range from 20 to 34 years). Three of the four study subjects were on hydroxyurea therapy at the time of evaluation.

Treatment Plan

The study was an open-label trial to evaluate the safety, and pharmacodynamics of eptifibatide in patients with SCD in the steady, non-crisis state. The study was divided into

four phases: a screening phase; a treatment phase; a post-treatment phase; and a follow-up phase. Eligible study subjects were admitted to the General Clinical Research Center (GCRC) one day prior to commencement of study drug administration. On the day of study drug administration, two baseline samples (i.e., time –30 min) for platelet aggregation studies and plasma CD40L were obtained using a 21-gauge needle. Beginning immediately thereafter, each subject received two 180 μg/kg boluses of Eptifibatide 10 minutes apart, followed immediately by a continuous infusion at 2 μg/kg/min for a total of 6 hours. Throughout this treatment phase, safety assessments were obtained by monitoring vital signs, clinical laboratory test results (complete blood counts, PT/PTT and renal and liver function tests), and any observed or reported adverse events. The post-treatment phase began immediately after the eptifibatide infusion was completed and lasted for a total of 24 hours. Seven days after completion of the study-drug infusion, each subject returned as an outpatient for the follow-up phase, which involved a thorough history and physical examination, a detailed assessment for adverse events, clinical laboratory safety tests, and final plasma samples for pharmacodynamic analyses.

Platelet aggregation

The pharmacodynamics of Eptifibatide in this patient population was analyzed by performing platelet aggregation studies on platelet rich plasma samples taken prior to the study drug infusion, immediately following discontinuation of the drug, and at the 7-day post-infusion follow-up. For all platelet aggregation studies, the blood samples were drawn into 1.2 mM PPACK, rested for 15 min at 37ºC, then centrifuged at 200*g* for 15 minutes before the red and white blood cells were removed. Aggregation was then measured in platelet-rich plasma

by an optical aggregometer (Chrono-Log, Havertown, PA) using autologous platelet-poor plasma as a reference and either 20μM ADP or 5μM TRAP6 as agonists.

CD40L, beta-thromboglobulin (βTG) and platelet factor 4 (PF4) measurement

Peripheral blood samples were collected by venipuncture from the antecubital vein via a 21-gauge needle into 0.13M sodium citrate. To separate plasma from blood cells, samples were centrifuged at 200*g*, PGI_2 (1U/mL) was then added to plasma before removing platelets at a 750*g* centrifugation, and microparticles at a 16,000*g* centrifugation. Each centrifugation was 15 minutes in duration and was preceded by a 15-30 minute rest of platelets at 37ºC. According to the manufacturer's recommendations, a fibrin clot was formed by the addition of 1U thrombin/mL plasma. The resulting defibrinated plasma was stored at -80ºC until it was analyzed. CD40 ligand, βTG and PF4 levels were measured in thawed plasma with ELISA kits (Alexis Biochemicals, San Diego, CA and American Diagnostica, Greenwich, CT, respectively). Both assays rely on antibody capture techniques and horseradish peroxidase conjugates. Reactions are developed using a tetramethylbenzidine developing solution.

Luminex Assays

Plasma levels of inflammatory cytokines were measured using luminex MAP technology.[32] Briefly, specific fluorescent microspheres were used to recognize target analytes in human plasma samples. Dual lasers identified microspheres and allowed the amount of analyte bound to be quantified. Assays were performed by Rules Based Medicine (Houston, TX).

Flow cytometry

Platelet exposure of P-selectin and CD40L were measured by whole blood flow cytometry as described previously.[33] Briefly, 5 μl of whole blood obtained from 0.13M sodium citrate anti-coagulated blood was incubated with either FITC-conjugated anti-CD62P and PE-conjugated anti-CD40L or appropriate fluorophore- and isotype- matched control antibodies (BD PharMingen, San Diego, CA). Samples were incubated at room temperature for 30 minutes, fixed with 0.7% paraformaldehyde, and read by a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Platelets were selected based on characteristic forward- and side-scatter profiles.

Results

Safety

Eptifibatide was well tolerated by the study subjects. The patients did not experience any side-effects that were thought to be secondary to the administration of eptifibatide. One subject complained of 2 days of a sore throat and a non-productive cough on his day 7 follow-up visit that was thought to be due to a viral upper respiratory tract infection. There were no bleeding manifestations or clinically meaningful changes in any of the safety laboratory studies (hematological, biochemical, or coagulation parameters) that were evaluated before, during and following eptifibatide infusion.

Platelet granule release and aggregation to leukocytes in SCD plasma is not increased by eptifibatide treatment

Despite the reduction in platelet:platelet aggregation, eptifibatide has been reported to increase some markers of platelet activation and the degree of platelet:leukocyte aggregation.[34] Therefore, platelet activation markers β-thromboglobulin (β-TG) and platelet factor 4 (PF4) were measured to determine if eptifibatide increases platelet granule release in patients with SCD. Neither β-TG nor PF4 were increased by eptifibatide treatment (Figure 12A). Furthermore, surface expression of platelet P-selection remained unchanged in the study subjects as a result of eptifibatide treatment (Figure 12B). Blood cells simultaneously positive for the leukocyte marker CD45 and the platelet marker GPIX were analyzed by whole blood flow cytometry to determine the amount of platelet:leukocyte aggregates in SCD patient samples. The high degree of platelet:leukocyte aggregates found in the blood of patients with SCD was not increased by treatment with eptifibatide, remaining near constant for all time points measured (Figure 12C). These results suggest that the already high level of platelet granule release and aggregation to leukocytes that exists in SCD is not increased by treatment with eptifibatide.

Elevated CD40L in SCD plasma is reduced by eptifibatide treatment

Soluble CD40L levels in cell-free SCD plasma were measured. Six hours following eptifibatide infusion, soluble plasma CD40L levels were decreased in 3 of the 4 patients studied. CD40L levels remained unchanged for 1 patient (Figure 13). On average, soluble CD40L levels were reduced by approximately 35% in those patients whose plasma CD40L were decreased following eptifibatide treatment (Figure 13B). Given that higher levels of

soluble CD40L may be predictive of vascular inflammation,[35] these results suggest that eptifibatide treatment may be beneficial in reducing vascular inflammation and the risk of adverse cardiovascular events in patients with SCD.

The profile of inflammatory indicators in SCD plasma is altered by eptifibatide treatment

Plasma from patients with SCD was assayed for expression of inflammatory indicators before and after treatment with eptifibatide. Myoglobin, an indicator of damage to muscle tissue, was reduced by an average of 35%, macrophage inflammatory protein-1 alpha (MIP-1α) expression was reduced by 37%, and tumor necrosis factor alpha (TNFα) was reduced by 32% (Figure 14A). Conversely, eptifibatide treatment increased matrix metalloproteinases MMP-2 and MMP-9 by an average of 34% and 81%, respectively (Figure 14B). The adipokine leptin was similarly increased by an average 70% in plasma from SCD patients following eptifibatide treatment (Figure 14C).

Eptifibatide inhibits platelet aggregation in SCD patients

The inhibition of *ex vivo* platelet aggregation by eptifibatide was examined in patients with SCD. Platelet response to adenosine diphosphate (ADP) and thrombin receptor activating peptide (TRAP) was measured in all 4 study subjects at baseline, 6-hours, 24-hours, and 7-days post eptifibatide infusion. Eptifibatide significantly inhibited platelet aggregation in all 4 subjects studied (Figure 15). We found ADP-induced platelet aggregation to be inhibited by approximately 90% immediately following eptifibatide infusion (6-hours post infusion). Aggregation induced by varying concentrations of TRAP was inhibited by approximately 46-67%. Aggregation returned to normal at the 24-hour time point for all agonists.

Discussion

Sickle cell disease is characterized by increased thrombin generation, abnormal activation of fibrinolysis, increased platelet activation and decreased levels of anticoagulant proteins.[1] Additionally, increased levels of soluble tissue factor (TF) combined with TF-expressing endothelial cells and microparticles[36,37] constitute a hypercoagulable state of multifactorial etiology. The quagmire of vaso-occlusive events is compounded by an abnormal RBC membrane phospholipid asymmetry,[7,38] the adherence of sickle RBCs to vascular endothelium,[2] as well as ischemia-reperfusion injury.[3] In addition, SCD is an inflammatory state, as evidenced by chronic elevation of leukocyte counts, abnormal activation of granulocytes and monocytes[4,5] and chronically elevated levels of multiple inflammatory mediators.[2]

Platelet hyperactivity and increased release of the inflammatory mediator CD40L characterize the vascular pathogenesis of SCD just as it does in other inflammatory vascular diseases including ACS. Antagonism of platelet integrin αIIbβ3 by eptifibatide has proven to be safe and effective for the treatment of ACS, with patients benefiting from the inhibition of platelet aggregation and CD40L release. Improved clinical status of ACS patients due to Eptifibatide treatment was seen despite *in vitro* reports of granule release and platelet:leukocyte aggregation resulting from occupation of αIIbβ3 by the antagonist. Eptifibatide may prove to be beneficial in the treatment of SCD as well. However, the unique hemodynamics of SCD may make these patients less tolerant of residual αIIbβ3 activation caused by eptifibatide than the normal volunteers and ACS patients studied in

previous safety trials. Here we show that eptifibatide can be safely administered to SCD patients, that eptifibatide treatment may promote a favorable alteration of cytokine expression in SCD, and that the beneficial effects of decreased platelet aggregation and CD40L release are still achieved.

Eptifibatide was well tolerated by SCD patients. No adverse events were reported during the study, and there were no significant changes in any of the hematological, chemical, or coagulation parameters measured as a result of Eptifibatide infusion. Furthermore, SCD patients receiving Eptifibatide did not show increased platelet granule release as a result of drug treatment (Figure 12). Although elevated beyond normal levels, platelet activation markers β-TG and PF4 were not further increased by Eptifibatide treatment of the SCD patients in this study (Figure 12A). Similarly, platelet exposure of P-selectin remained relatively constant following Eptifibatide infusion for all time points studied (Figure 12B). Platelet:leukocyte aggregates were also not increased in SCD patients following eptifibatide treatment (Figure 12C). Collectively, these results suggest that antagonism of platelet integrin αIIbβ3 by eptifibatide does not increase platelet activation in the context of SCD and may be suitable for use in the treatment of SCD.

We also demonstrate in this study that soluble CD40L is reduced by eptifibatide treatment in 75% of SCD patients tested (Figure 13). Reductions averaged 35% in these patients (Figure 13B), suggesting that in addition to its safe administration, eptifibatide treatment may lower CD40L levels in SCD patients. As elevated CD40L is a potential indicator of vascular

inflammation[39] and may correlate to the SCD clinical state,[24] reductions in CD40L levels are likely to be beneficial to SCD patients.

Indeed, multiple inflammatory cytokines were reduced following eptifibatide treatment (Figure 14). As MIP-1α and TNFα are potent mediators of inflammation, reduced expression of these cytokines might result in beneficial effects for the SCD patient. Furthermore, a decrease in the injury indicator myoglobin may be further evidence of a therapeutic benefit of eptifibatide treatment for SCD patients. Upregulation of the matrix metalloproteinases MMP-2 and MMP-9 (Figure 14B) is consistent with vessel dilation, as is an increase in the adipokine leptin (Figure 14C).

Pharmacodynamic studies of eptifibatide have largely focused on its short-acting, reversible decrease in platelet aggregation. However, benefits to patients receiving eptifibatide are significant even a year following treatment.[40] One-year hazard ratios demonstrate a decreased risk of death and myocardial infarction following eptifibatide treatment,[41] suggesting that a one-time dose of the αIIbβ3 antagonist has long lasting effects. Since eptifibatide inhibition of platelet aggregation in SCD patients was comparable to results achieved in ACS treatment (Figure 15), SCD patients could presumably enjoy lasting benefit from eptifibatide treatment as well.

This study demonstrates that eptifibatide is safe for use in SCD patients. Furthermore, these data demonstrate that the effective inhibition of αIIbβ3-mediated platelet aggregation in SCD is comparable to that shown in ACS. Additionally, 75% of these patients demonstrated a

decrease in CD40L levels. As eptifibatide infusion led to a favorable alteration in the inflammatory profile of the plasma of these SCD patients, eptifibatide may have additional benefits for SCD patients. These results suggest that eptifibatide may be an effective treatment modality for the vascular occlusion that occurs in patients with SCD. Future studies designed to establish the efficacy of eptifibatide in SCD are therefore warranted.

Figure 12

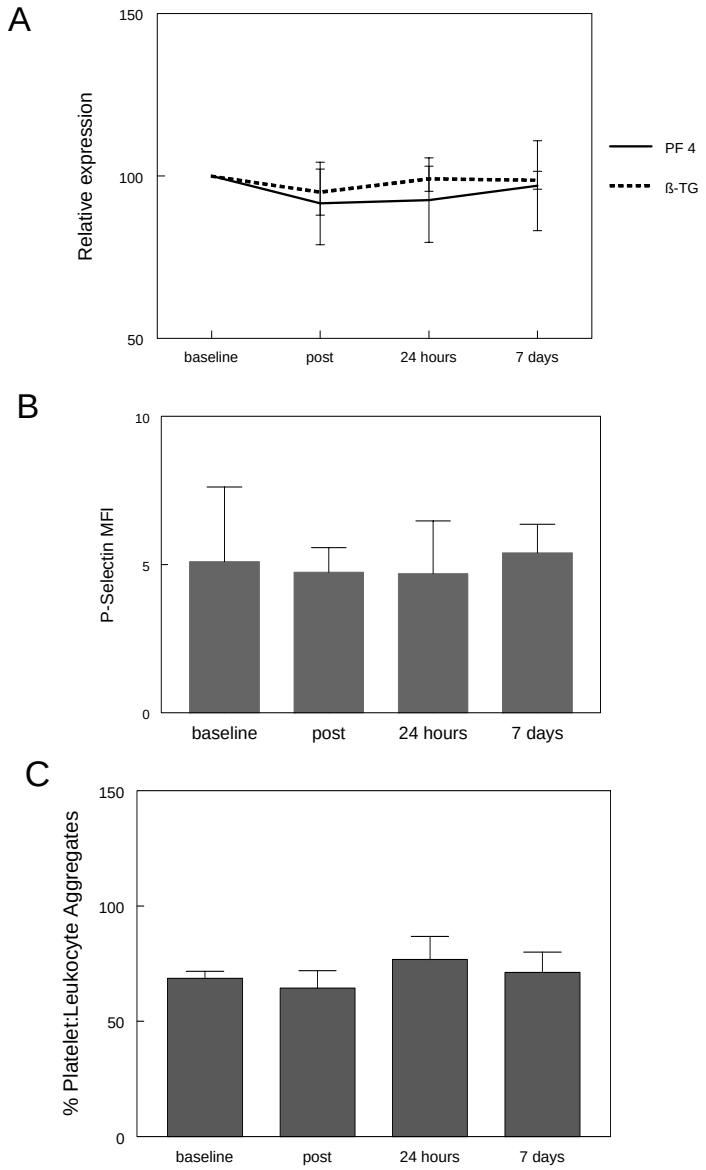

Figure 12. Eptifibatide treatment did not increase platelet activation in SCD plasma.
(A) Platelet activation markers beta-thromboglobulin (β-TG) and platelet factor 4 (PF4) were measured by ELISA in cell-free SCD plasma at the indicated time points. Baseline measurement of β-TG in SCD plasma averaged 193.9 IU/ml ± 7.7 IU/ml. Plasma levels of β-TG were unchanged by Eptifibatide treatment. Similarly, the average PF4 level of 77.8 IU/ml ± 10.9 IU/ml remained the same following Eptifibatide treatment. Results shown were normalized relative to baseline. (B) Surface expression of platelet P-selectin was analyzed by whole blood flow cytometry. Mean fluorescence intensity (MFI) of the P-selectin fluorophore remained relatively unchanged as a result of Eptifibatide treatment. (C) Whole blood from SCD patients was analyzed by flow cytometry. Leukocytes and platelets were selected by CD45 and GPIX specificity, respectively. Co-incidence of the leukocyte and platelet markers indicate that the amount of platelet:leukocyte aggregates in SCD patient samples was not increased by Eptifibatide treatment.

Figure 13

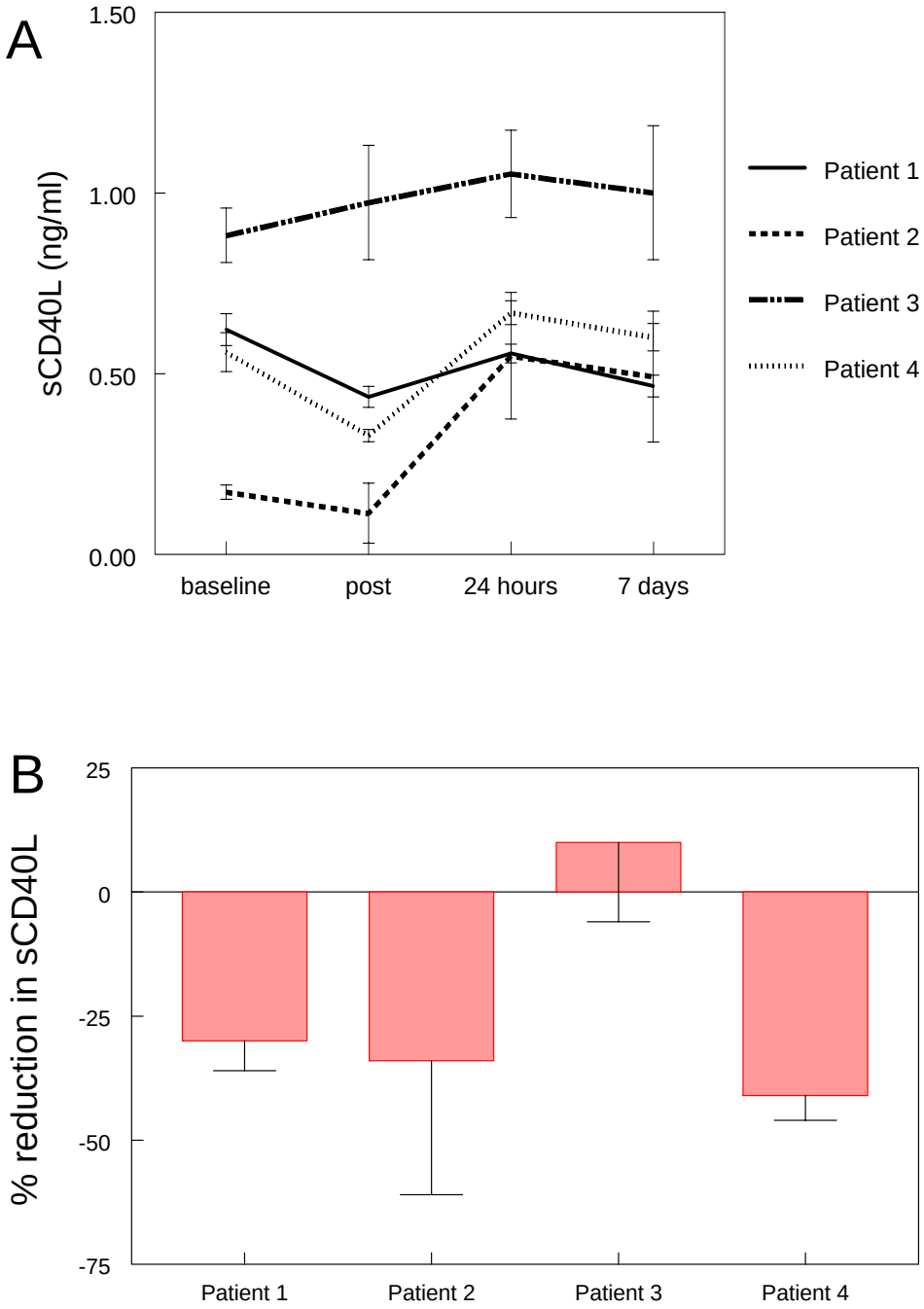

Figure 13. Elevated CD40L in SCD plasma is reduced by eptifibatide treatment
CD40L levels were measured in cell-free SCD plasma collected at time points indicated. For SCD patients 1, 2 and 4, ELISA results demonstrate a reduction in soluble CD40L at the 6h time point. Levels of plasma CD40L returned to baseline by the 24h time point. Soluble CD40L remained unchanged for SCD patient 3. (B) Eptifibatide treatment reduced CD40L levels by an average 35% for SCD patients 1, 2 and 4 as shown for the 6h time point. Results shown represent mean ± SEM.

Figure 14

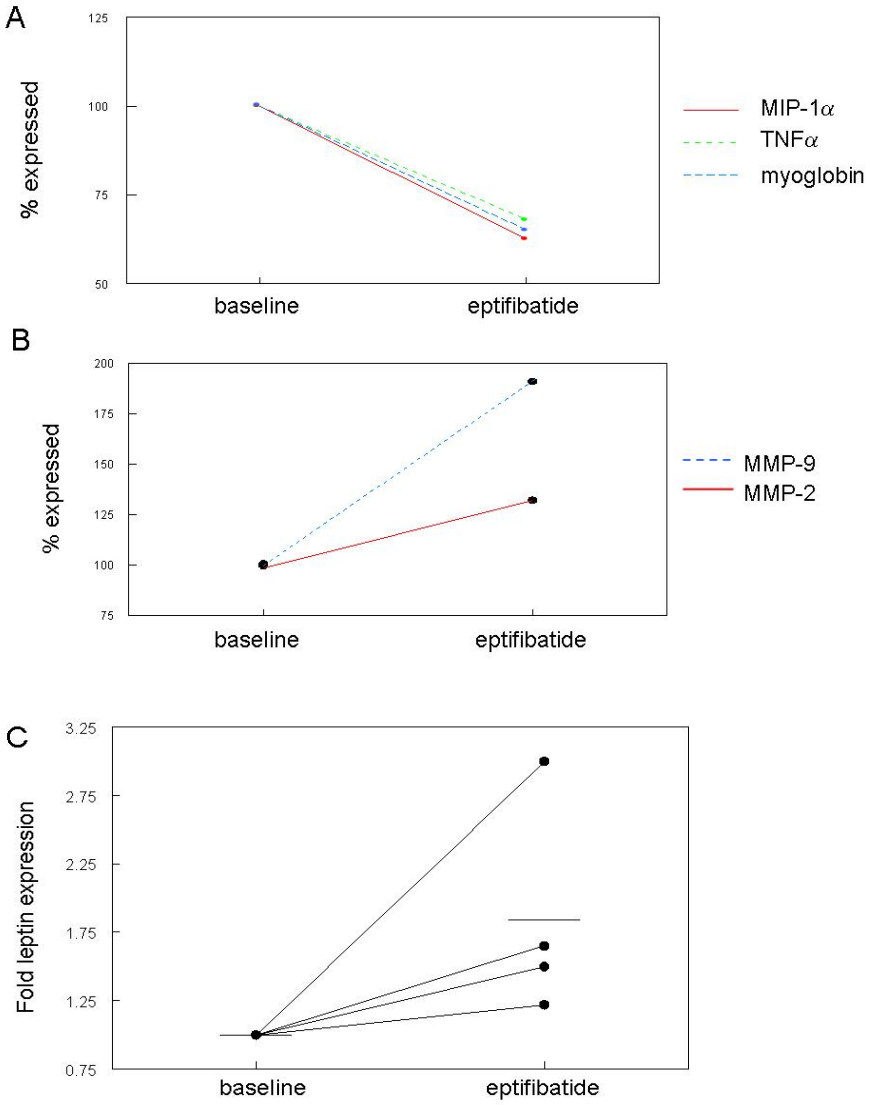

Figure 14. The profile of inflammatory indicators in SCD plasma is altered by eptifibatide treatment. (A) Average plasma concentration of myoglobin dropped from 6.9 ng/ml to 4.1 ng/ml, MIP-1α dropped from an average 12.3 pg/ml to 8.6 pg/ml, and average TNFα concentration was reduced from 7.0 pg/ml to 4.6 pg/ml. Reductions in myoglobin, MIP-1α and TNFα following eptifibatide infusion averaged 35%, 37% and 32%, respectively, for each patient. (B) Matrix metalloproteinases MMP-2 (334 ng/ml to 434 ng/ml) and MMP-9 (448 ng/ml to 596 ng/ml) increased average concentrations 34% and 81%, respectively, in SCD patient plasma following eptifibatide infusion. (C) Leptin concentrations increased from 0.78 ng/ml to 1.04 ng/ml following eptifibatide infusion, an average 1.84-fold increase in expression. Data shown determined by Luminex multiplex analysis of EDTA-anticoagulated patient plasma.

Figure 15

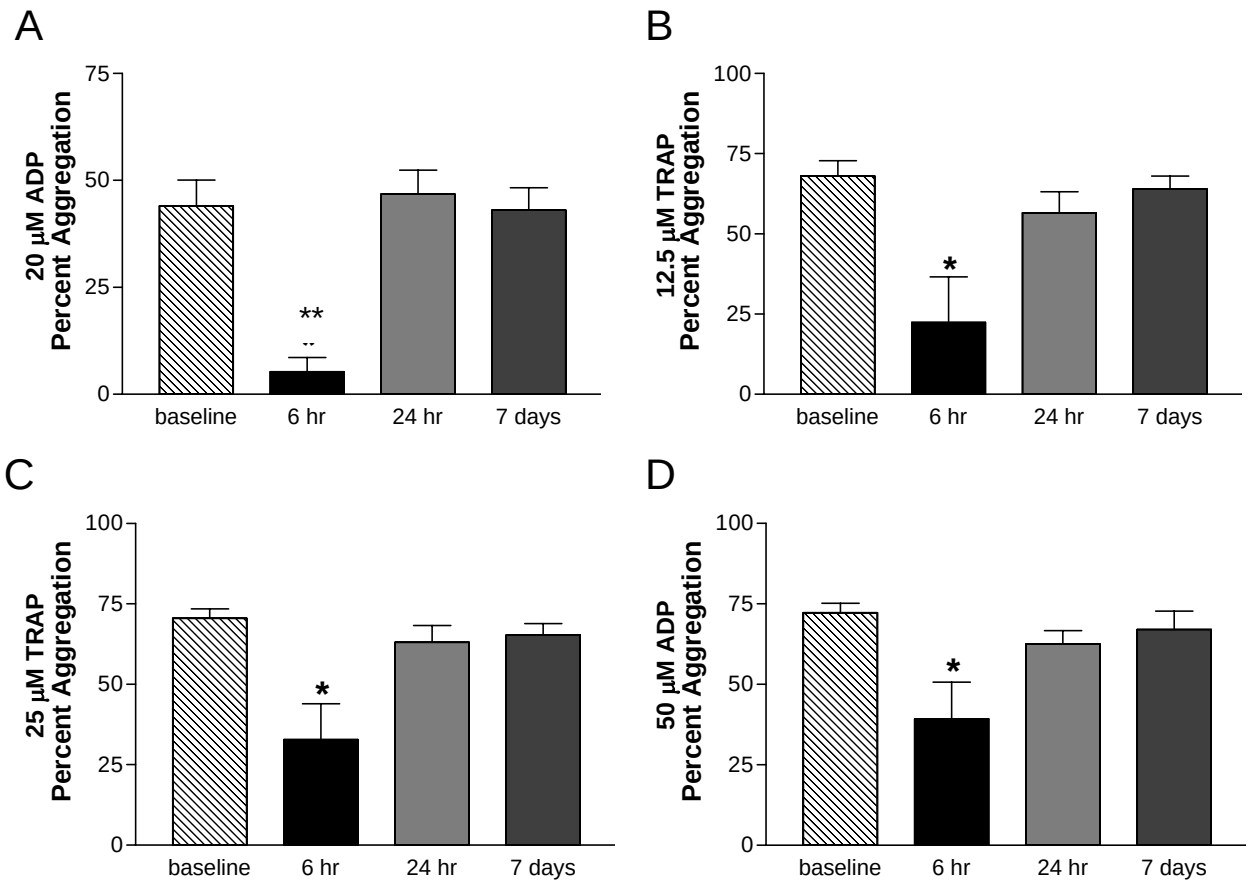

Figure 15. Eptifibatide inhibits platelet aggregation in SCD patients

The extent of ex vivo platelet aggregation inhibition with eptifibatide was examined in patients with SCD disease. Platelet response to ADP and TRAP was measured in four steady state, homozygous SCD patients at baseline, 6-hours, 24-hours, and 7-days post eptifibatide infusion. (A) Eptifibatide inhibited platelet aggregation to 20 μM ADP by approximately 90%, **$p<0.001$. (B-D) Platelet aggregation was inhibited ~67% in response to 12.5 μM TRAP*, ~54% in response to 25 μM TRAP*, and ~46% in response to 50 μM TRAP, *$p<0.05$. Aggregation profiles returned to baseline levels within 24 hours and were unchanged 7 days post eptifibatide infusion.

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CHAPTER V.

CONCLUSIONS

CD40L mediates chronic inflammation in multiple disease states[1]. B cell proliferation,[2] endothelial adhesion molecule expression,[3] and procoagulant activity through tissue factor upregulation[4] are all mechanisms by which CD40L exerts its inflammatory effects. All of these indices of inflammation characterize SCD,[5] but by unknown mechanisms. SCD is also characterized by increased platelet activity, suggesting that the vast amount of CD40L stored in platelets[6] are prone to surface exposure and release as soluble fragments into the bloodstream.[7] Therefore, CD40L might be more available and mediating the chronic inflammation that occurs in SCD patients. Prior to this work, the role of platelets and the inflammatory protein CD40L in SCD pathology had not been clearly established. Here we present evidence that platelets participate in the chronic inflammation of SCD via CD40L and provide new insights into mechanisms contributing to SCD pathology.

Just as sCD40L levels are elevated in a variety of diseases involving vascular inflammation,[8-10] we found that sCD40L is elevated in SCD as well. Indeed, our findings indicate an average 30-fold elevation of sCD40L in SCD versus normal plasma. We also demonstrate a corresponding decrease in the amount of CD40L stored in the platelets of SCD patients, more than enough to account for the increased plasma amounts measured. The

elevated sCD40L in SCD plasma positively correlates with increased TF and participates in the induction of TF, VCAM-1 and ICAM-1 expression via its interaction with CD40. Biological activity of sCD40L in SCD plasma is further confirmed by its induction of B cell proliferation. Together, these data identify sCD40L as potentially important for both inflammation and coagulation in SCD, and suggest a previously unrealized participation of platelets in SCD pathogenesis.

In vivo studies of the organ pathology in SCD provide further evidence of the role the potent inflammatory mediator CD40L plays in this disease. Following bone marrow transplantation to convey the SCD phenotype to mice, the effects of anti-CD40L treatment or the lack of endothelial CD40 or β3 integrin were examined. In the SCD mice where CD40L activity was inhibited, there was significantly less lung, liver and kidney damage as shown by immunohistochemical staining for iron and fibrin deposits. Even more impressively, the spleens of anti-CD40L treated mice appeared normal compared to the disrupted architecture and grossly enlarged spleens typical of SCD. Furthermore, the lack of CD40 was protective against the accumulation of lung pathology in the BMT SCD mice. These results demonstrate a role for CD40L in the development of organ damage in SCD, and suggest that blocking CD40L activity could reduce the damage that accumulates in the vital organs of SCD patients.

Platelet hyperactivity and increased release of the inflammatory mediator CD40L characterize the vascular pathogenesis of SCD just as it does in other inflammatory vascular diseases including ACS. Antagonism of platelet integrin αIIbβ3 by eptifibatide has proven

to be safe and effective for the treatment of ACS, with patients benefiting from the inhibition of platelet aggregation and CD40L release. Improved clinical status of ACS patients due to eptifibatide treatment was seen despite *in vitro* reports of granule release and platelet:leukocyte aggregation resulting from occupation of αIIbβ3 by the antagonist. Eptifibatide may prove to be beneficial in the treatment of SCD as well. However, if eptifibatide occupancy of αIIbβ3 causes residual outside-in platelet activation, the unique hemodynamics of SCD may make these patients less tolerant of residual platelet activity than the normal volunteers and ACS patients studied in previous safety trials. Here we show that eptifibatide can be safely administered to SCD patients. Furthermore, these data demonstrate that the effective inhibition of αIIbβ3-mediated platelet aggregation in SCD is comparable to that shown in ACS. Additionally, 75% of these patients demonstrated a decrease in CD40L levels. As eptifibatide infusion led to a favorable alteration in the inflammatory profile of the plasma of these SCD patients, eptifibatide may have additional benefits for SCD patients. These results suggest that eptifibatide may be an effective treatment modality for the vascular occlusion that occurs in patients with SCD. The extent to which eptifibatide, or other platelet antagonist, may be effective as a treatment modality for SCD should be determined.

Together, these data provide new insights into mechanisms of inflammation in SCD. The findings presented here suggest that platelet CD40L plays an important role in the inflammatory processes of SCD and in the resulting pathology. Furthermore, these results suggest a mechanistic link by which the mutated hemoglobin of sickle RBC leads to the numerous downstream effects of inflammation, vascular occlusion and hypercoagulation. The process of sickling damages the RBC of SCD such that membrane leakiness releases

ADP[11-13] and phospholipid asymmetry exposes phosphatidylserine.[14] Therefore, sickle RBC can be irritants,[15] provoking a variety of effects that include platelet activation.[11] Activated platelets expose CD40L to their surface[16] and release soluble CD40L fragments.[7]

When CD40L is no longer encrypted in platelets, the multifunctional inflammatory mediator can cause a host of proinflammatory and procoagulant effects.[3] B cell proliferate,[17] VCAM-1 and ICAM-1 expression is upregulated,[18] and tissue factor production is increased;[19,20] all in response to CD40 ligation. Increased leukocyte counts, endothelial activation, and procoagulant activity are SCD characteristics associated with increased vascular occlusion[21] and more clinically severe SCD,[22,23] with an increased propensity for the sickling of slowly transversing RBC.

A model for chronic inflammation in SCD is proposed here (Figure 16) where we present graphically how this self-perpetuating process predisposes SCD patients to recurring and ongoing inflammation. As presented here, this repetitive cycle requires both platelet activation and CD40L activity. Therefore, platelets and CD40L are identified as two potential therapeutic targets to interrupt the chronic inflammatory cascade and treat the manifestations of SCD.

This research goes on to suggest that a particular platelet antagonist, eptifibatide, may be beneficial to SCD patients. Pending more extensive studies, interventional therapy with the αIIbβ3 antagonist could provide lasting benefits for SCD patients just as it does for patients with ACS.[24] If this or other mechanisms aimed at antagonizing platelet activation and

reducing CD40L activity are employed in SCD, these patients could potentially enjoy longer life spans, less pain crises, fewer hospitalizations, and overall better health as a result.

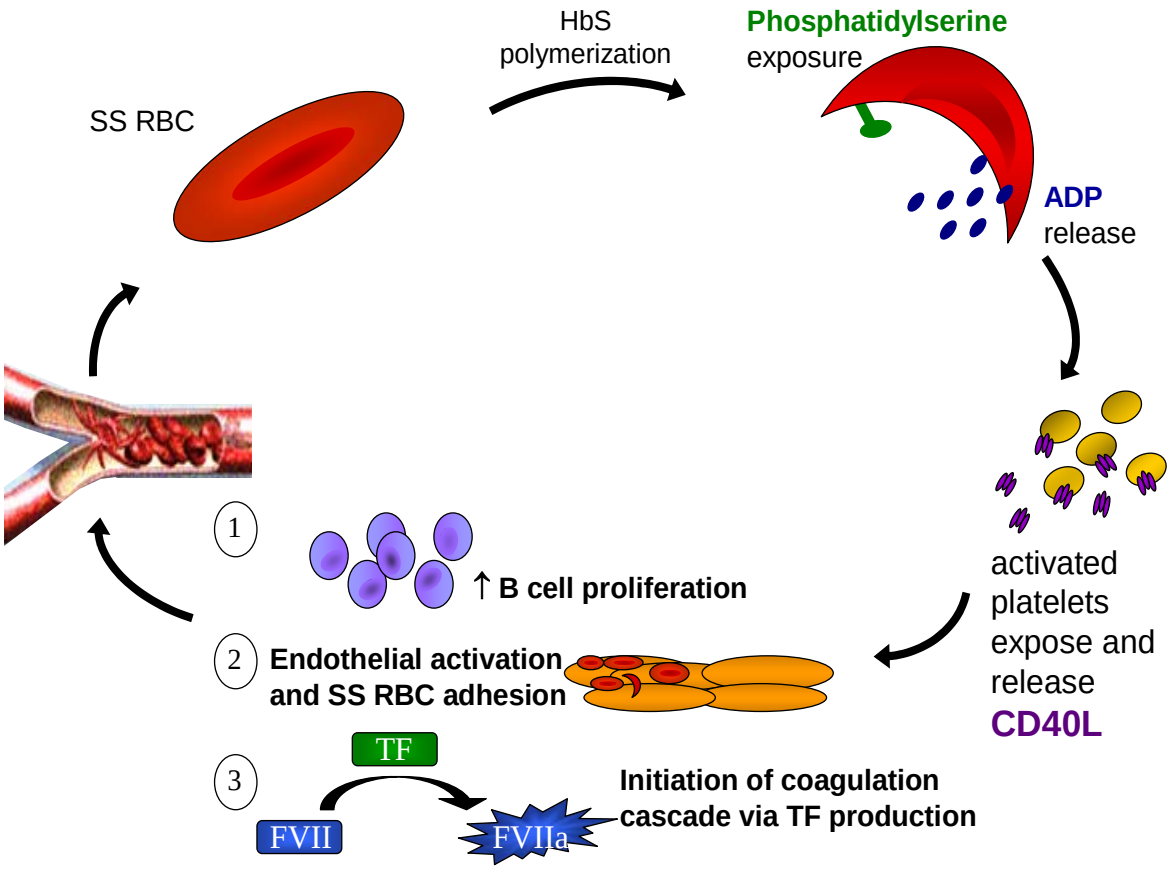

Figure 16. Potential model of platelet CD40L role in chronic inflammation in SCD. Hemoglobin S abnormally polymerizes upon deoxygenation, causing distorted, sickle-shaped RBC. Repetitive sickling damages RBC membranes, causing phosphatidylserine exposure and the release of ADP. Platelets in turn become activated, exposing and releasing CD40L. CD40L mediates B cell proliferation, endothelial activation, and TF production. These processes increase the propensity for vascular occlusion. RBC transit through the vasculature is slowed, oxygenation is delayed, sickling is increased and a persistent cycle of chronic inflammation ensues.

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