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Targeting iNKT Cells for the Treatment of Sickle Cell Disease

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Abstract

Sickle cell disease (SCD) causes widely disseminated vaso-occlusive episodes. Building on evidence implicating invariant NKT (iNKT) cells in the pathogenesis of ischemia/reperfusion injury, recent studies demonstrate that blockade of iNKT cell activation in mice with SCD reduces pulmonary inflammation and injury. In patients with SCD, iNKT cells in blood are increased in absolute number and activated in comparison to healthy controls. iNKT cell activation is reduced by agonists of adenosine 2A receptors (A_{2A}Rs) such as the clinically approved coronary vasodilator, regadenoson. An ongoing multi-center, dose-finding and safety trial of infused regadenoson, has been initiated and is providing preliminary data about its safety and efficacy to treat SCD. Very high accumulation of adenosine may have deleterious effects in SCD through activation of adenosine 2B receptors that are insensitive to regadenoson. Future possible therapeutic approaches for treating SCD include selective A_{2B}R antagonists and antibodies that deplete iNKT cells.

Keywords

iNKT cells; sickle cell disease; regadenoson

Introduction

Sickle cell disease (SCD) is the most common genetic disease among African Americans, affecting approximately 1 in 400 births in the United States. The basis for SCD is a non-synonomous mutation in the 6th position of the β globin gene (Glu→Val) that causes de-oxygenated sickle hemoglobin (ααSS) to polymerize, resulting in rigid sickle shaped erythrocytes. Historically, the pathogenesis of SCD has been attributed to deformed sickle erythrocytes that mechanically obstruct capillaries to cause microvascular occlusion (Hebbel et al., 1981). Emerging evidence, however, shows that vaso-occlusion is a complex, mutli-cellular process involving endothelial and platelet activation, a pro-coagulant state, leukocyte adhesion to endothelial cells and ischemia-reperfusion injury (IRI).

Vaso-occlusion secondary to IRI is the pathogenic basis for the two most common episodic morbidities in SCD, pain [1] and acute chest syndrome (ACS)[2]. End organ damage in

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people with SCD is due in part to recurrent episodes of disseminated microvascular IRI. Therapeutic options for pain and/or ACS episodes in the acute setting are currently limited to supportive care with fluids, antibiotics, opioids and, in the case of ACS, blood transfusion. Additional therapies for hospitalized patients with pain and ACS are needed. Hydroxyurea has been used successfully to increase the production of fetal hemoglobin and reduce red cell sickling [3]. Since data from several studies indicate that white cell and platelet activation accompanies red cell sickling and hemolysis, new strategies to dampen the frequency or severity of vaso-occlusive episodes include interrupting leukocyte adhesion to endothelial cells with intravenous immunoglobulins [4] or selectin inhibitors [5].

Murine models of sickle cell vaso-occlusion have provided preliminary evidence that IRI contributes to a profound pro-inflammatory environment causing leukocyte activation, migration and adhesion, thus sustaining and propagating vaso-occlusion initiated by sickled RBCs. Although the mechanism IRI in vaso-occlusion has not been fully elucidated, recent evidence implicates invariant NKT (iNKT) cells as key to propagating an inflammatory cascade associated with IRI. In this review we will examine evidence suggesting that iNKT cells contribute to IRI, vaso-occlusion and ultimately end organ damage in people with SCD. In a murine model of SCD, adenosine 2A receptor ($A_{2A}R$) agonists have shown promise for interrupting iNKT cell activation and reducing pulmonary morbidity. We describe efforts that are currently underway to extend these findings to patients with SCD.

Role of inflammation in Sickle Cell Disease

SCD causes sterile inflammation

SCD disease triggers hemolysis, ischemia and tissue necrosis that evoke sterile inflammation that is thought to be triggered by a combination of nitric oxide depletion [6] production by damaged cells of damage-associated molecular patterns (DAMPs) [7], ATP, ADP, cytokines [8] and lipid mediators [9]. A heightened state of inflammation in SCD is evidenced by increased levels of leukocytes, platelets, cytokines (TNF-α, IL-1, IL-6, IL-8) and leukotrienes (B_4 and E_4). Kaul et al. [10;11] postulated that during vaso-occlusion, recurrent episodes of hypoxia followed by reoxygenation and exposure to oxidant species contributes to inflammation and promotes further vaso-occlusion. In SCD mice, hypoxia/reoxygenation increased leukocyte adhesion and emigration and increased oxidant production in vascular endothelial cells. Intravital microscopy analyses in SCD mice indicate that sickle RBCs interact primarily with adherent leukocytes (WBCs) in postcapillary and collecting venules leading to vascular obstruction [12]. A pan selectin inhibitor, GMI1070, inhibits RBCleukocyte interactions, and results in improved microcirculatory blood flow and improved survival [5]. Evidence of vascular injury includes increased circulating endothelial cells and soluble circulating adhesion molecules (ICAM-1, VCAM-1, E-selectin and P-selectin). ATP and ADP released from activated or damaged cells activates platelets via two G protein coupled ADP receptors (P2Y1 and P2Y12) and via ATP through the ligand-gated P2X1 receptor [13]. Platelet activation in SCD is associated with increased secretion of thrombospondin, β-thromboglobulin, platelet factor 4, and increased expression of P-selectin, glycoprotein IIbIIIa and CD40 ligand. Low nitric oxide levels in SCD probably contribute to platelet activation [14]. Platelet activation is associated with increased platelet adhesion to microvascular endothelium [15] and formation of platelet heteraggregates with erytorocytes [16] and leukocytes including neutrophils, monocytes and eosinophils [17]. In mouse and human blood, neutrophil activation is partly due to the formation of P selectin-mediated platelet-neutrophil heteroaggregates. Multi-spectral imaging identified a subpopulation of activated neutrophils with adhered platelets that exhibit greater oxidative activity than singlet neutrophils (Figure 1) [17]. Both the fraction of neutrophils with adhered platelets, and the number of platelets bound per neutrophil are increased as a result of human or murine SCD. Among individuals with SCD, increased

WBC count is associated with a higher incidence of ACS, pain, silent and hemorrhagic strokes and premature death. Levels of soluble adhesion molecules ICAM-1 and VCAM-1 correlate with risk for pulmonary hypertension, and high leukotriene (B_4 and E_4) levels have been associated with an increased incidence rate of ACS and pain.

A limited number of clinical studies have examined therapies to decrease inflammation and potentially improve the course of vaso-occlusive pain and ACS. In separate randomized clinical trials, corticosteroids were shown to significantly decrease length of hospital stay; however, many of the participants treated with corticosteroids experienced rebound vaso-occlusion and up to 25% required re-hospitalization [18]. Thus, no anti-inflammatory therapy is currently standard treatment for vaso-occlusive episodes.

iNKT cells mediate IRI

A_{2A} agonsits have also been found to reduce injury following ischemia or trauma in many tissues, including liver [19;20;21;22;23;24], kidney [25;26;27;28], skin [29], lung [30;31;32], heart [33;34;35;36], intestine [37] and spinal cord [38;39;40]. The cellular targets of A_{2A}Rs initially were not clear. Neutrophils and macrophages express A_{2A}Rs that respectively inhibit oxidative burst and adhesion molecule expression [41] and cytokine production [42]. Reutershan et al. utilized mice with *loxp* sites flanking the first coding exon of the A_{2A}R gene, *adora2a*, and crossed these mice to LysMCre mice. All lines were made congenic to C57BL/6J using marker-assisted selection. In LysM-Cre × $A_{2A}R^{f/f}$ mice that selectively lack A_{2A}Rs in neutrophils and macrophages, A_{2A}R activation was still highly effective at reducing liver or lung IRI [43]. Adoptive transfer of $CD4^+$ (but not $CD8^+$ T cells) to $Rag1^{-/-}$ mice reconstituted severe injury from IRI [44]. The A_{2A} agonsit ATL146e inhibited this injury if the transferred cells had A_{2A}Rs, but not if they lacked A_{2A}Rs [35]. This result is striking because $Rag1^{-/-}$ mice reconstituted with $A_{2A}R^{-/-}$ $CD4^+$ T cells have a normal complement of A_{2A}Rs in all cells except the reconstituted T cells. The results indicate that despite the widespread distribution of A_{2A}Rs on platelets and leukocytes, A_{2A} agonists reduce IRI primarily by their effects on T cells.

In 2005 Shimamura et al. [45] found that liver reperfusion injury was associated with an expansion and activation of NKT cells. Subsequently, Lappas et al. found that depletion of NKT and NK cells with PK136, an antibody that binds to NK1.1 found only on NKT and NK cells, or blockade of CD1d-restricted iNKT cell activation with an anti-CD1d antibody produces protection from liver IRI that is equivalent to and not additive with protection by ATL146e [46]. These studies indicate that the adenosine-sensitive T cells that mediate IRI are iNKT cells. The mechanisms by which iNKT cells are activated in IRI are not entirely clear, but recent studies suggest that tissue injury may result in the formation of a galactose-containing glycolipid that can activate the invariant TCR [9]. In addition, iNKT cell activation may be facilitated by the binding of phosphatidylserine on the surface of apoptotic cells to T cell Ig-like mucin-like-1 (TIM-1) receptors on NKT cells [47].

Role of iNKT cells in SCD

To determine whether iNKT cells play a role in SCD tissue damage, Wallace et al. compared the lungs of wild type and NY1DD mice. Pulmonary iNKT cells from NY1DD mice are increased in number and activated compared to C57BL/6 mice [48]. NY1DD lung iNKT cells displayed significantly increased levels CD69 and IFN-γ compared to C57BL/6 mice. The % of pulmonary iNKT cells positive for IFN-γ increased from 5% in wild type mice to 37% in NY1DD mice, a difference of 7.4-fold. Interrupting iNKT cell activation or migration into the lungs reduced pulmonary inflammation and improved pulmonary function in NY1DD SCD mice.

Wallace et al. discovered that there are high levels of IFN-γ in iNKT cells derived from NY1DD mouse lungs [48]. FACS analysis of pulmonary lymphocytes for cell surface CXCR3 revealed that the expression CXCR3 is significantly higher (% positive cells) on CD4 T-cells (6-fold), CD8 T-cells (7-fold), NK cells (4-fold), and iNKT cells (2-fold) from NY1DD mice than C57BL/6 controls. ELISAs of pulmonary tissue homogenate also revealed significantly increased levels of IFN-γ and the IFN-γ inducible chemokines CXCL9 and CXCL10 in lungs of NY1DD mice as compared to C57BL/6 mice [48]. Neutrilization of CXCR3 was found to significantly reduce numbers of PMNs, $CD4^+$ cells, $CD8^+$ cells, NK cells and NKT cells in the lungs of NY1DD mice. Furthermore, anti-CXCR3 treated NY1DD animals had significantly decreased vascular leak and increased arterial oxygen saturation as compared to NY1DD mice. Treatment of NY1DD mice with anti-CXCR3 antibodies significantly improved breathing parameters. These finding suggest that iNKT cells orchestrate an inflammatory cascade by involving IFN-γ and INF-γ-inducible chemokines. Hence, blocking CXCR3 signaling constitutes another potential therapeutic approach to treating SCD.

iNKT cells are the primary targets of $A_{2A}R$ activation in SCD

Wallace et al. reasoned that since $A_{2A}R$ activation inhibits the activation of iNKT cells and other leukocytes and platelets, that $A_{2A}R$ activation would reduce SCD lung injury. Administration of ATL146e for 3 days by subcutaneous Alzet minipumps produced a dose-dependent reduction in the number of lung iNKT cells, NK cells and neutrophils [49]. The optimal dose, 10 ng/kg/min, is similar to the optimum noted in liver, kidney and heart models of IRI, and is below the threshold dose that changes heart rate and blood pressure. When 10 ng/kg/min of ATL146e is continued for 3 days and then discontinued, lung function is improved on day 3 and somewhat on day 4, but reverts to baseline by day 7. This experiment demonstrates that the palliative effects of ATL146e on lung function in SCD does not desensitize over at least 3 days, nor is there a rebound effect after the compound is discontinued. However, the reduction in injury cause by $A_{2A}R$ activation is gradually reversed after the agonist is discontinued.

Since $A_{2A}Rs$ are found not only on iNKT cells, but also on most leukocytes and platelets and on various other tissues, Wallace et al. sought to determine the relative importance of A_{2A} receptors on various cells in mediating lung protection in SCD. In order to evaluate the role of $A_{2A}Rs$ on iNKT cells we crossed NY1DD mice with SCD to $Rag1^{-/-}$ mice that lack mature T cells including NKT cells. NY1DD × $Rag1^{-/-}$ mice displayed reduced pulmonary injury that was restored by adoptive transfer of 10^6 purified iNKT cells. Reconstituted lung injury was reversed by the $A_{2A}R$ agonsit, ATL146e, unless the adoptively transferred iNKT cells were pretreated with the $A_{2A}R$ alkylating antagonist, 5-amino-7-[2-(4-fluorosulfonyl)phenylethyl]-2-(2-furyl)-pryazolo[4,3-]-1,2,4-triazolo[1,5-c]pyrimidine (FSPTP) which completely prevented protection [49]. These data indicate that despite the widespread expression of $A_{2A}Rs$ on most cells of the immune system, activation of $A_{2A}Rs$ on iNKT cells is required for effective inhibition of pulmonary injury by $A_{2A}R$ agonsits in the NY1DD mouse model of SCD. The pivitol role of $A_{2A}Rs$ on iNKT cells may be related to the fact that $A_{2A}R$ mRNA is induced on these cells when they are activated as a consequence of SCD [49].

Activation of iNKT cells in patients with sickle cell disease

There is an expansion and activation of iNKT cells in patients with SCD compared to healthy African American controls (Figure 2) [49]. Given the general leukocytosis of patients with SCD, it is notable that there is selective expansion of iNKT cells among lymphocytes, from < 1% in control blood, to an average of 6% in the blood of SCD patients.

We also examined the activation state of iNKT cells in human blood. SCD causes a significant increase in the % iNKT cells positive for CXCR3 and CD69, and an increase in intracellular IFN-γ. On the basis of these data we concluded that SCD produces a similar expansion and activation of iNKT cells in mice and patients.

Deleterious effects of adenosine

Although adenosine accumulation in SCD can produce beneficial effects by activating anti-inflammatory signaling in iNKT cells and other leukocytes and platelets, very high accumulation of adenosine that may occur during severe vaso-occlusive episodes can activate other adenosine receptor subtypes and may produce deleterious effects. For instance, activation of renal A_1 receptors can reduce glomerular filtration and produce a potentially damaging anti-diruesis [50]. In addition, in the severe Berkeley mouse model of SCD, activation of A_{2B} receptors has been implicated as contributing priapism [51], penile fibrosis [52] and red blood cell sickling [53]. It remains to be determined how frequently adenosine accumulation that is sufficient to activate A_1 and A_{2B} receptors occurs in patients with SCD. Fortunately, regadenoson, which is being tested as an $A_{2A}R$ agonist to treat SCD, is selective for the $A_{2A}R$ over the A_1 or A_{2B} subtypes. It should be possible to find a therapeutic dose of regadenoson that activates anti-inflammatory A_{2A} receptors without producing detrimental effects that might be produced by activating other adenosine receptor subtypes. It will be interesting in the future to consider combinations of A_{2A} agonists and A_{2B} antagonists as a strategy to optimally prevent and treat vaso-occlusive events.

Clinical trial of the A_{2A} agonist, regadenoson

Our group is currently conducting a phase I safety study of regadenoson in adults and children with SCD. Regadenoson is a selective $A_{2A}R$ agonist that is FDA approved for myocardial perfusion imaging in individuals unable to undergo adequate exercise stress. The strategy of testing an $A_{2A}R$ agonist to treat SCD is based on the reduction of pulmonary inflammation and injury we observed after inhibiting iNKT cell function in murine models of SCD with CD1d-blocking antibodies or $A_{2A}R$ agonists. Among the challenges of administering regadenoson to patients with SCD for the purpose of reducing inflammation is to determine the optimal dose and duration of treatment. For cardiac stress tests, regadenoson is administered as a 400 μg bolus over 10 seconds and produces coronary vasodilation and, not infrequently, transient hypotension and tachycardia. The rationale for using regadenoson as an $A_{2A}R$ agonist is that the anti-inflammatory effects of $A_{2A}Rs$ are more potent than the cardiovascular toxicities. Since the ultimate goal is to administer regadenoson during a pain or ACS episode, regadenoson's short half-life necessitates that we administer the drug as a continuous infusion. In the absence of dosing guidelines for a biological meaningful effect on iNKT cells, we estimated dosing and cardiovascular toxicities from animal studies. Based on binding to recombinant human A_{2A} adenosine receptors we determined that regadenoson is about 15 times less potent than ATL146e (the $A_{2A}R$ agonist used in animal studies). However, the terminal half life of regadenoson in man is about 12 times longer than ATL146e (2 hours vs. 10 min), and during continuous infusion, regadenoson is expected to reach steady state blood levels about 12 time higher than ATL146e. Hence we estimated that regadenoson and ATL146e will have similar potencies during infusions and will both achieve maximally effective anti-inflammatory effects in the range of 10 ng/kg/min. We also estimated that the threshold for cardiovascular side effects will be about 100 ng/kg/min.

An ongoing study of regadenoson in SCD is comprised of 4 stages. In stage 1, we will determine the maximally tolerated and biologically effective dose of regadenoson during a 12-hour infusion. Stage 2 will determine the safety of a 24 hour infusion. Stages 3 and 4 will

examine the safety of regadenoson in adults and children with SCD, respectively, during a vaso-occlusive episode. iNKT cell activation markers will be measured before, during and after the infusion. If we determine a safe and biologically effective dose, we will pursue studies to determine whether regadenoson is efficacious for the treat of pain episodes or ACS. In the future we anticipate that in additional to $A_{2A}R$ activation, other means of depleting or inhibiting the activation of iNKT cells will be utilized to prevent or treat acute exacerbations of SCD. In addition, inhibition of platelet and neutrophil activation may also prove to be effective.

Targeting iNKT cells in future clinical trials of SCD

A_{2A} agonists such as regadenoson represent one approach to transiently inhibiting the activation of iNKT cells. There are a number of additional approaches that could be tried in the future to produce a more persistent blockade. It may be possible to disrupt activation of human iNKT cells with antibodies that target CD1d. Although such antibodies have recently been shown to activate antigen presenting cells to produce pro-inflammatory responses [58], this approach may still be effective in SCD patients. Another strategy is to deplete iNKT cells using antibodies that target the human invariant TCR [54]. For use in SCD it will be important to demonstrate that such antibodies do not produce transient iNKT cell activation. Finally, it may be possible to selectively inhibit iNKT cell activation through the use of glycolipid antagonists that bind to CD1d but do not activate iNKT cells [55]. These approaches will require careful investigation because of growing evidence that iNKT cells play an important role in immune resistence to certain pathogents [56;57].

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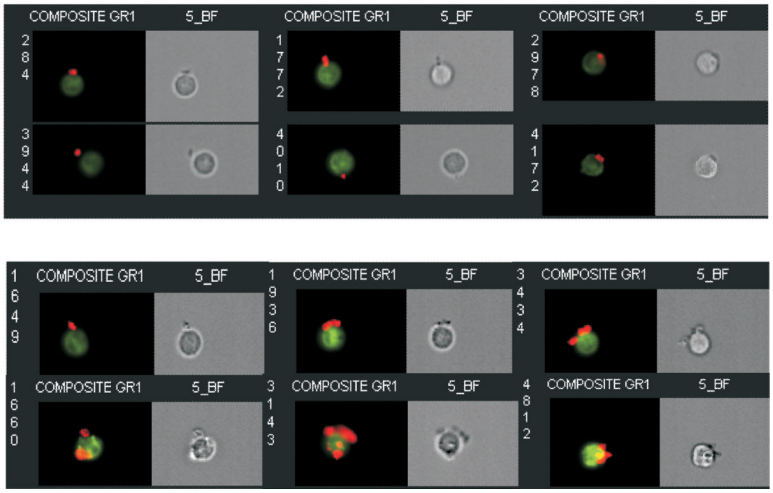

Figure 1. Effect of SCD on platelet-neutrophil aggregates in mouse blood

Blood was collected from mice without and with SCD and analyzed to determine the % of neutrophils with 1 or more adhered platelets, and the average number of adhered platelets/ neutrophil in heteroaggregates. Platelet-neutrophil aggregates were imaged by Multi-spectral Imaging Flow Cytometry using an ImageStream™ flow cytometer (Amnis Corporation, Seattle, WA). Images of platelet-neutrophil aggregates in blood derived from wild type (top) and SCD mice (bottom) indicates that SCD increases the number of platelets (red) bound/ neutrophil (green). Reprinted from [17].

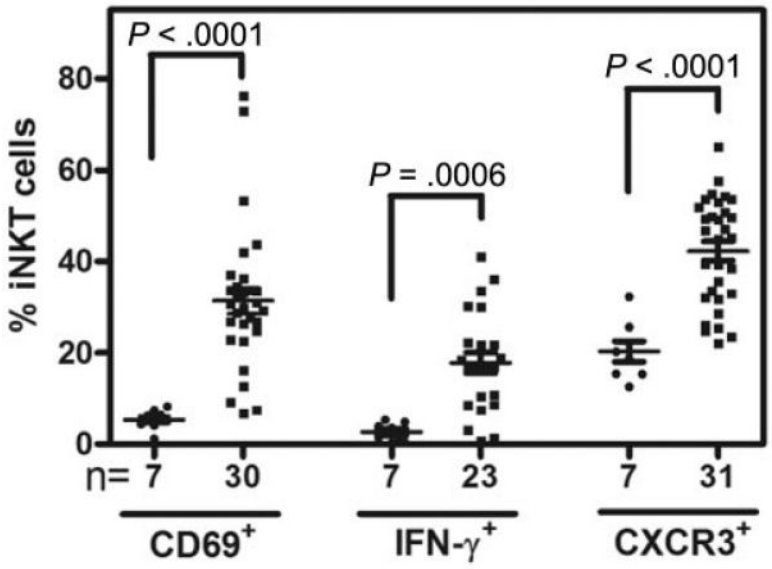

Figure 2. Effects of SCD on human iNKT cells

Blood from patients with HbSS SCD or African American controls was collected into EDTA and analyzed by flow cytometry. Each *point* show iNKT cell activation markers in controls (●) or individuals with HbSS at baseline (■). Reproduced from [49].