Chapter

Model Human β Thalassemic Erythrocytes: Effect of Unpaired Purified α-Hemoglobin Chains on Normal Erythrocytes

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Abstract

β thalassemias arise from genetic defects that interfere with the synthesis of the β hemoglobin chain and the subsequent production of the normal $\alpha_2\beta_2$ hemoglobin tetramer. As a consequence of this decreased β-chain synthesis, unpaired α-hemoglobin chains are found within the red blood cell (RBC). The unstable α-chains are associated with a number of cellular defects, including: membrane-bound globin; membrane thiol oxidation; altered cytoskeletal proteins; decreased cellular and membrane deformability; and increased susceptibility to both endogenous and exogenous oxidants. Surprisingly, while significant injury to human thalassemic RBC arise from the unpaired α-chains, the underlying intra-RBC mechanisms are not easily studied in patient samples or in mouse models. To better study the *fate of excess α-chains* in human RBC, the model β Thalassemic cell was developed. Model human β thalassemic RBC is made by entrapping purified human α-chains within normal RBC via osmotic lysis and resealing. This human model allows for the systematic examination of the mechanisms underlying the α-chain mediated damage in the β thalassemic RBC. Studies utilizing the model β thalassemic RBC have demonstrated that the α-chains give rise to an iron and glutathione-dependent, self-amplifying and self-propagating oxidative reaction.

Keywords: β thalassemia, α-hemoglobin chains, iron, red blood cell, erythrocyte, oxidation, free radicals, glutathione, deformability

1. Introduction

The thalassemias are a major cause of morbidity and mortality throughout much of the world [1–9]. Thalassemias are characterized by the disruption of the synthesis of normal adult hemoglobin (HbA; an $\alpha_2\beta_2$ tetramer; **Figure 1**) consequent to a diverse array of genetic mutations/deletions to either the β or α-hemoglobin chain genes (Chromosomes 11 and 16, respectively). As a consequence of reduced/absent production of β-chains, β thalassemia is characterized by the presence of highly unstable monomeric α-chains as these chains cannot self-associate and indeed require a chaperone protein to prevent precipitation [10]. In contrast, α thalassemia is characterized by the presence of relatively stable tetrameric β chains. Interestingly, as schematically shown in **Figure 1**, unlike most genes, there are four copies of the α-globin genes;

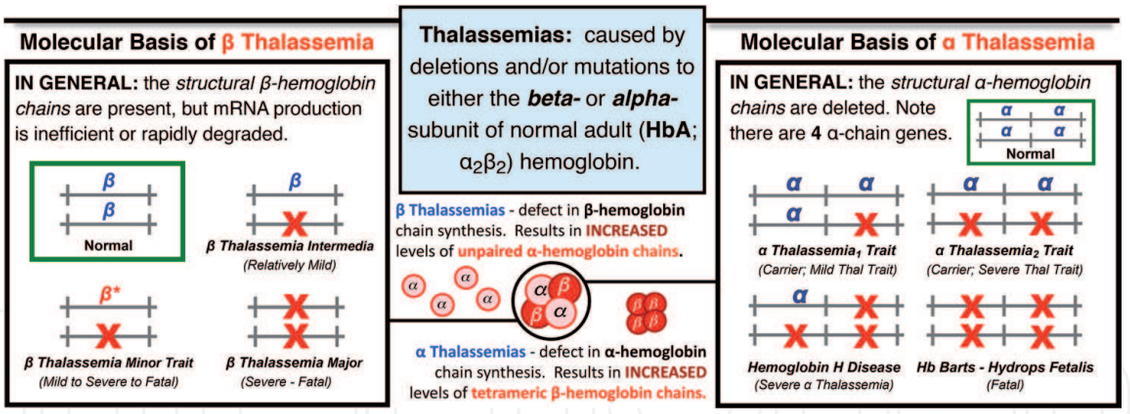

Figure 1.
Molecular basis and clinical diagnosis of the α and β thalassemias [1–9]. β denotes abnormal β hemoglobin gene such as HbS or HbE.*

this is in contrast to the expected two copies of the β-globin genes. The evolutionary duplication of the human α-chain genes may have been favored consequent to the inherent instability of monomeric α-chains. Indeed, the instability of the α-chains is the key factor underlying the pathophysiology of the β thalassemic red blood cell (RBC). Moreover, the pathophysiology of β thalassemia can be further complicated by the geographical prevalence, and high frequency, of a number of mutated β hemoglobin genes (e.g., sickle hemoglobin, hemoglobin E and hemoglobin C). If a mutated β-chain is the only functional β-chain present, the resultant disease will be more severe than that observed in β thalassemia Intermedia (a single normal β-globin gene). Loss of both β-chain genes gives rise to severe β Thalassemia Major which is fatal in the absence of transfusion therapy. The α thalassemias are characterized by a broader range of disease states due to the presence of 4 α genes. The loss of expression from a single gene (α Thalassemia$_1$ Trait) is often asymptomatic and undiagnosed; though the individual is a carrier for α Thalassemia and, in high frequency geographic areas may be at elevated risk for symptomatic disease transmission to an offspring. Deletion of two or three α-genes results in severe disease as a single active α-gene cannot, due to the instability of the chain, produce sufficient mature α-chains to form sufficient HbA. Loss of all four α-genes is fatal (resulting in Hydrops fetalis) due to the crucial role that α-chains play in embryonic and fetal hemoglobin. In contrast to β thalassemia, stable mutated α-chains are rare so typically these do not pose a significant complication in the pathophysiology of α thalassemia.

In this chapter we will further explore the pathophysiology of the β thalassemic RBC. Surprisingly, while significant injury to the thalassemic erythrocyte arises from the excess α-chains, the underlying mechanisms by which these chains damage and subsequently destroy the thalassemic RBC in the bone marrow and peripheral blood have not been clearly delineated. Our lack of understanding of the mechanisms of α-chain mediated damage is due, in part, to three major factors: (1) studies of RBC from β thalassemic individuals are difficult to do since these cells, upon collection, already exhibit significant injury and represent a survivorship bias since up to 80% of erythroid precursors are destroyed within the bone; (2) β thalassemic patients are typically transfused to both correct the severe anemia accompanying the disease and to prevent endogenous erythropoiesis of defective RBC; and (3) the lack of a good experimental model by which the pathophysiology of excess globin chains on human RBC can be examined.

While little can be done to change the first two problems, researchers have attempted to tackle the third issue using murine models of thalassemia [11–17]. Original murine studies examining the knockout of the murine β-chains were not

productive as the murine α-chains behave significantly different from their human counterpart. To overcome this problem, human α-chain genes were inserted into the mouse genome in place of the murine genes. Again, these studies failed to give rise to as severe a phenotype as is seen in the human disease. Subsequent studies utilized additional mutations to produce symptomatic disease in the murine context—albeit with still substantial differences from the pathophysiology seen in the human β thalassemic RBC. Hence, an alternative approach for studying the pathophysiology of unpaired α-chains on the human RBC was needed.

2. Model human β thalassemic RBC

To this end, our laboratory developed an *in vitro* model of the HUMAN β thalassemic erythrocyte [18–27]. In this model, purified human α-chains are entrapped within normal human RBC (or, if desired, mouse RBC) by osmotic lysis and resealing (**Figure 2**) [18–34]. As previously shown, osmotic lysis and resealing results in RBC exhibiting normal hemoglobin concentration and volume (**Table 1**) as well as normal ATP concentration, oxidant sensitivity, morphology and

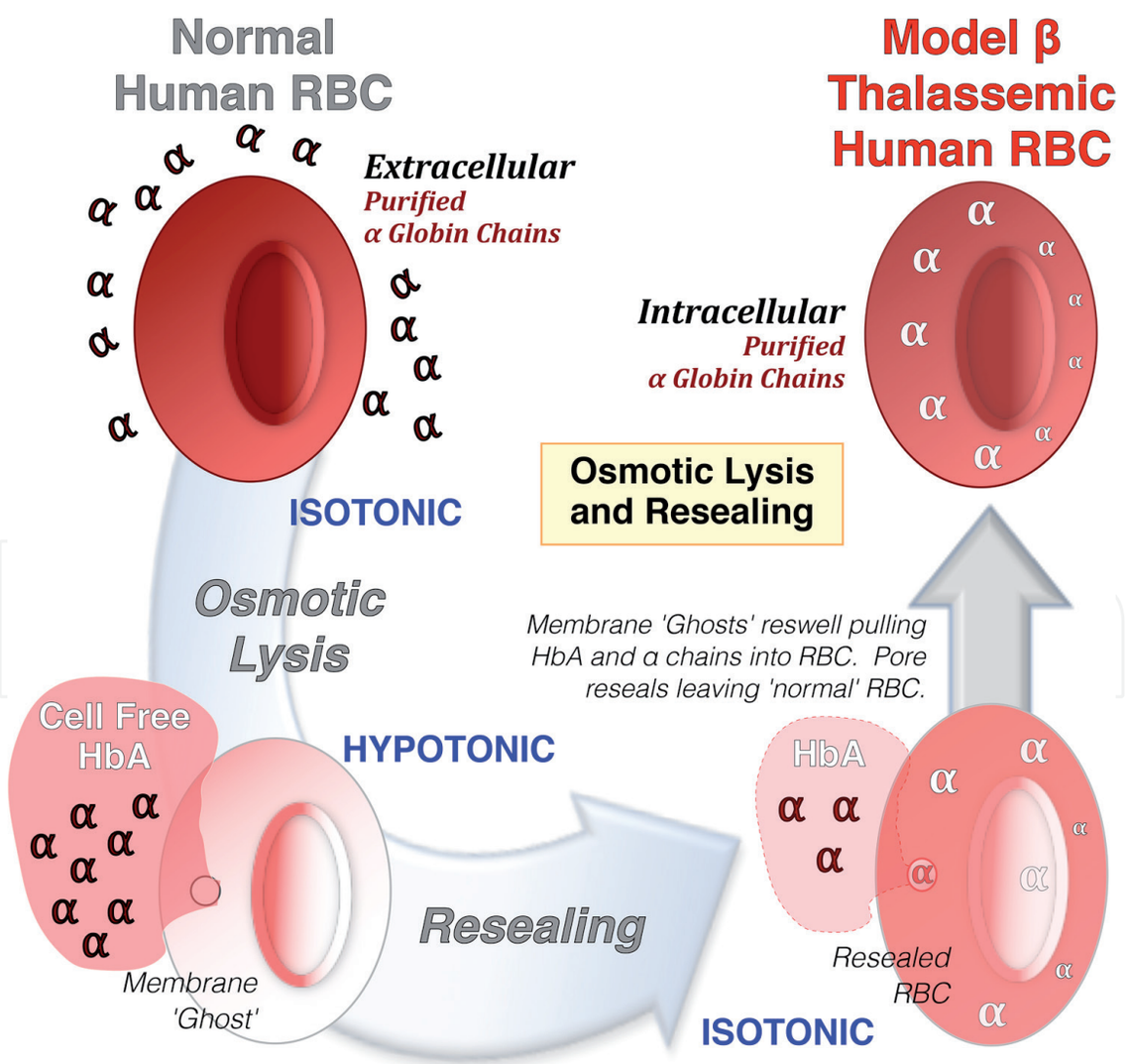

Figure 2.
Generation of model β thalassemic RBC from normal human donor cells via osmotic lysis and resealing [18–34]. Osmotically lysed and resealed RBC have normal morphology and metabolism and exhibit normal in vivo *survival.*

Parameter	N	Normal	Resealed*	Model β thal*	Model α thal*,**
Mean cell volume (μm^3)	7	88.9 ± 1.5	85.8 ± 4.5	78.6 ± 0.8	76.0 ± 4.0
Mean cell hemoglobin (pg)	7	30.5 ± 0.8	28.5 ± 2.7	23.4 ± 0.6	26.9 ± 2.1
Mean cell Hb conc. (g/dl)	7	34.2 ± 0.7	33.2 ± 1.7	29.6 ± 0.6	35.5 ± 1.3
Red cell distribution width (RDW)	7	12.9 ± 0.2	17.5 ± 4.5	29.2 ± 0.5	21.3 ± 3.8

**Immediately post resealing and washing 3×.*
***Made via the entrapment of purified human β hemoglobin chains.*

Table 1.
Cellular characteristics of model β and α thalassemic RBC [18–27].

deformability while allowing for the efficient entrapment of exogenous compounds [18–34]. Indeed, studies with resealed human RBC demonstrate that this methodology can be used to correct enzyme deficiencies, enhance antioxidant levels, and be used *in vitro* to study malarial growth and maturation. Moreover, murine studies demonstrated that osmotically resealed murine RBC exhibited normal *in vivo* survival. To manufacture the *model β thalassemic* RBC from normal human donor cells, purified, heme-containing, α-hemoglobin chains were prepared by dissociation of CO-treated HbA in the presence of parahydroxymercuribenzoate, followed by ion exchange chromatography, to isolate the purified CO-α-chains as previously described [1, 18, 20–22, 35, 36]. Analysis of the purified α-chains by mass spectroscopy demonstrated the expected mass values for the α-globin chain. The purified α-chains can be stored at −80°C as carbon monoxide stabilized chains (CO-α-chains) and then thawed immediately prior to use.

Characteristic	β thalassemic RBC	Model β thalassemic RBC
Microcytic RBC (MCV <80 fl)	+	+ (progressive)
K+ Loss	+	+
Unpaired α-chains	1–3% of total hemoglobin in peripheral blood reticulocytes	3–4% of total hemoglobin
Membrane Bound Globin	Increased	Increased; correlated with α-chain autoxidation & heme release.
Membrane proteins/ thiols	Loss of spectrin and ankyrin. Oxidation of membrane thiol (-SH) groups.	Loss of spectrin and ankyrin correlated with membrane thiol (-SH) oxidation, α-chain autoxidation, and heme release.
Cellular deformability	Decreased cellular and membrane deformability	Decreased; correlated with iron/heme deposition in membrane.
Intracellular oxidant stress	Evidence supporting increased oxidative damage	Increased H_2O_2; correlated with α-chain autoxidation & heme release.
Oxidant susceptibility	Increased	Increased; correlated with membrane bound iron/heme.
Membrane bound iron/heme	Increased membrane associated hemoglobin & Heinz body formation	Increased membrane heme; correlated with α-chain autoxidation.
Short in vivo survival	+	+ (*murine model β Thal RBC*)

Table 2.
Comparison of the pathologic characteristics of β thalassemic and model β thalassemic erythrocytes [18–27].

Entrapment of the purified α-chains within normal erythrocytes is done by osmotic lysis and resealing as previously described [18–34]. Briefly, washed, packed erythrocytes (80–85% hematocrit) are mixed with the purified CO-α-chains (10 mg/ml packed red cells) and then placed as a thin film within 11.5 mm diameter dialysis tubing (MW cutoff of 3500). The samples are dialyzed against a hypotonic lysis buffer (5 mM potassium phosphate buffer and 2 mM EDTA; pH 7.4) at 4°C for 60 min. The dialysis tubing is then transferred to an isotonic resealing buffer (5 mM potassium phosphate, 160 mM NaCl, and 5 mM glucose; pH 7.4) with gentle agitation for 30 min at 37°C. Following resealing, cells are washed with saline until the supernatant is clear. Using this procedure, approximately 70–80% of the initial packed erythrocyte volume is recovered. Radiolabeled α-chains can be utilized to quantitate the intracellular entrapment [18, 20].

To determine whether the model β thalassemic erythrocytes exhibit the cellular abnormalities characteristic of true β thalassemic cells, a number of cellular parameters have been examined. The results of these studies demonstrate that the α-chain loaded erythrocytes exhibit structural and functional changes very similar to those seen in β thalassemic erythrocytes (**Table 2**). Consequently, this model allowed for the systematic examination of the mechanisms underlying the α-chain mediated damage within the β thalassemic RBC and to directly determine the ontogeny of the pathologic events underlying the RBC injury and to experimentally test potential therapeutic approaches.

3. Iron-glutathione driven progeria of the thalassemic RBC

Containing approximately 20 mM iron, the RBC is the most ferruginous somatic cell in mammals. Under normal conditions, most of this iron is complexed within hemoglobin (as heme) with virtually none present as free metal (i.e., non-heme). This near perfect compartmentalization of iron may, however, break down in certain pathologic states such as β thalassemia and sickle cell disease resulting in the autoxidation of hemoglobin (i.e., formation of methemoglobin and hemichromes). Of physiologic importance, the monomeric α-chains spontaneously autoxidize to

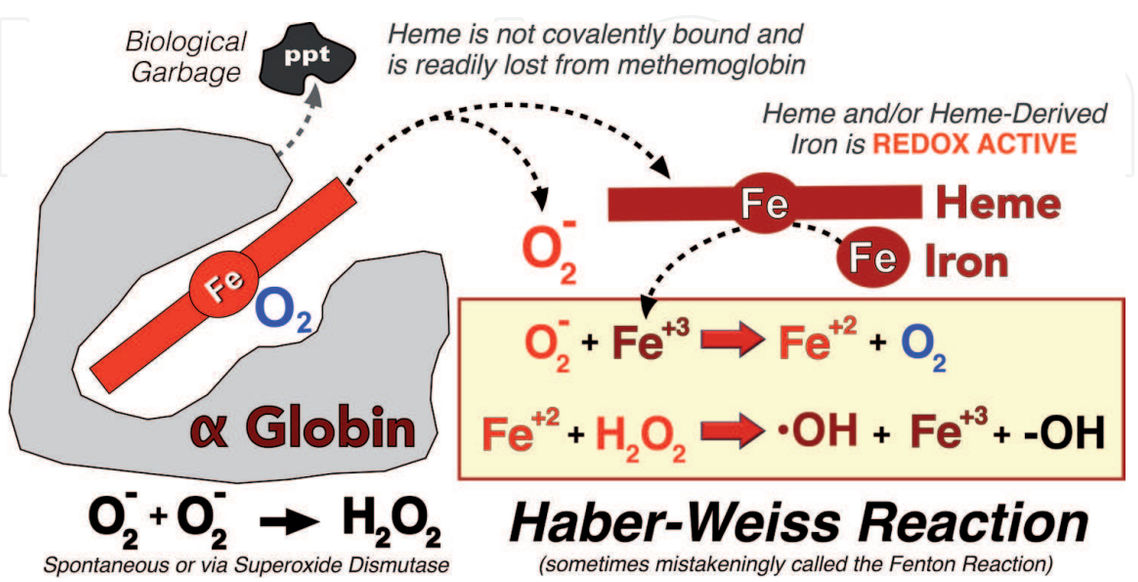

Figure 3.
Free radical basis of α-hemoglobin autoxidation and free radical injury. Consequent to the formation of methemoglobin, the non-covalently bound heme is 8-times more likely to escape the globin chain. Release of the heme initiates iron-dependent free radical reactions. References: [18–27].

methemoglobin, simultaneously generating superoxide (O_2^-), at a rate 8-times that of normal hemoglobin (**Figure 3**) [18, 20, 22–27, 37–39]. Moreover, consequent to the formation of methemoglobin, the non-covalently bound heme is more likely to escape the heme pocket of the globin chain giving rise to elevated levels of free, redox-active, intraerythrocytic iron. The O_2^- produced via the autoxidation of hemoglobin can reduce ferric (Fe^{+3}) to ferrous (Fe^{+2}) iron or form hydrogen peroxide (H_2O_2; either spontaneously or enzymatically via superoxide dismutase). Importantly, the iron, O_2^-, and H_2O_2 can, via the Haber-Weiss Reaction, give rise to the formation of the 'dreaded' hydroxyl radical (•OH) which rapidly reacts with virtually all biological constituents converting pristine materials into biological garbage (**Figure 3**). However, despite the general concept that free iron and the formation of free radicals are bad, the actual iron-dependent pathophysiology of the β thalassemic RBC has been poorly understood.

Mechanistically, studies using the model β thalassemic RBC have demonstrated that the initial autoxidation of the unpaired α-chains initiates an iron and reduced glutathione (GSH) dependent, self-amplifying and self-propagating reaction with the subsequent release of even more heme and, eventually, free iron. Schematically, this self-amplifying, self-propagating injury pathway is shown in **Figure 4**. As noted, the reaction process is initiated by the autoxidation of the unpaired α-chains which gives rise to the release of free heme and the generation of O_2^-. Interestingly, at this

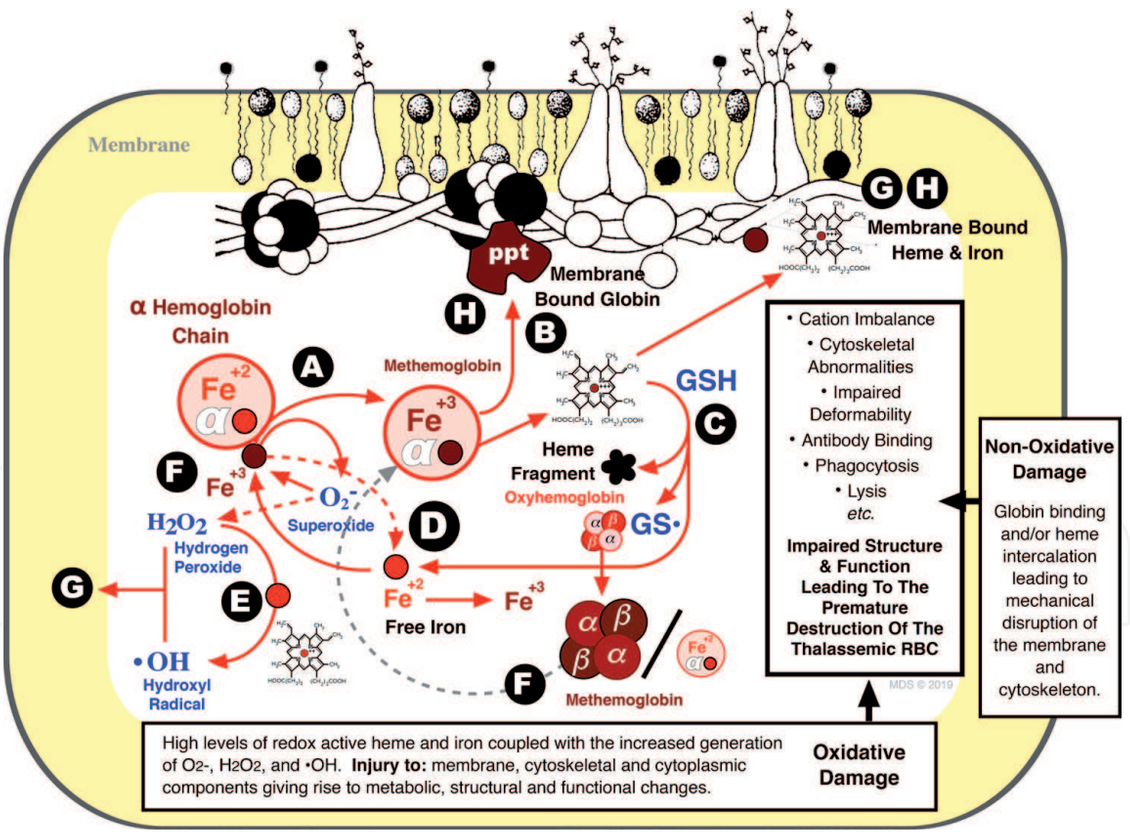

Figure 4.
Damage to multiple components of the β thalassemic RBC is mediated by an Fe-GSH dependent mechanism. As thalassemic RBC circulate, α-hemoglobin chains autoxidize (A) giving rise to superoxide (O_2^-) and methemoglobin [MetHb]. The heme moiety of the α metHb chains are released yielding free heme and globin (B). Free heme reacts with reduced glutathione [GSH; reaction (C)] resulting in cleavage of the heme group and the formation of a glutathione radical (GS•) and the release of free Fe^{+2} (D). The free iron (E) reacts with hemoglobin derived reactive oxygen species (ROS: O_2^- and H_2O_2) to generate •OH – A highly reactive radical capable of damaging all cellular components including cellular metabolism and cation/anion balance. The ferric iron (Fe^{+3}), in the presence of additional GSH or O_2^- regenerates Fe^{+2} which then oxidizes additional hemoglobin (F). Reactions (G) and (H) represent subsequent oxidative and non-oxidative injury to the cell. Oxidative pathway generated from references: [18–27, 40].

point, a key component of this pathway is GSH; an 'anti-oxidant' present at high intracellular (~2.3 mM) concentrations within the RBC [41, 42]. GSH readily reacts with free heme resulting in the cleavage of the heme ring, the release of free iron, and the formation of a thiol radical (GS•). This reaction leads to the rapid amplification of the oxidative damage to the RBC [18, 20, 22–27]. .The importance of the released iron and GSH was documented experimentally. As shown in **Figure 5A**, addition of Fe^{+3} to hemolysates from normal RBC results in the rapid oxidation of oxyhemoglobin in an iron-dose dependent manner. The oxidation of hemoglobin can be inhibited by the inclusion of an iron chelator (shown is deferoxamine; DFO) or by chemical depletion of GSH (not shown) [23, 27]. Moreover, in the intact model β thalassemic RBC, chemical depletion of GSH inhibited iron-driven hemoglobin oxidation (**Figure 5B**) [23, 27]. In contrast, as shown in **Figure 5C**, increasing the amount of intracellular GSH (via osmotic lysis and resealing; see **Figure 2**) in the model β thalassemic RBC significantly exacerbated injury to the cell. This enhanced injury is readily seen by the significantly reduced deformability (i.e., increased mean cell transit time) of the GSH-loaded model β thalassemic cells relative to the control model β thalassemic RBC. Importantly, as shown, GSH supplementation of normal RBC in the absence of iron had no detrimental effects on cellular deformability.

The enhanced oxidative stress, and consumption of GSH, was further noted by the time dependent decrease in GSH noted in the model β thalassemic RBC (**Table 3**) [27]. Moreover, a decrease in the NADPH/NADPtotal ratio was noted. NADPH is utilized to regenerate reduced GSH from oxidized (GSSG) glutathione via glutathione reductase. This decrease in the NADPH/$NADP_{total}$ ratio is likely reflective of both the high GSH-GSSG-GSH cycling but also of metabolic abnormalities arising consequent to iron and free radical mediated inhibition of normal glucose metabolism (**Figure 4**). Also of physiological importance, was the finding that the model β thalassemic RBC exhibited significantly ($p < 0.001$) decreased catalase activity

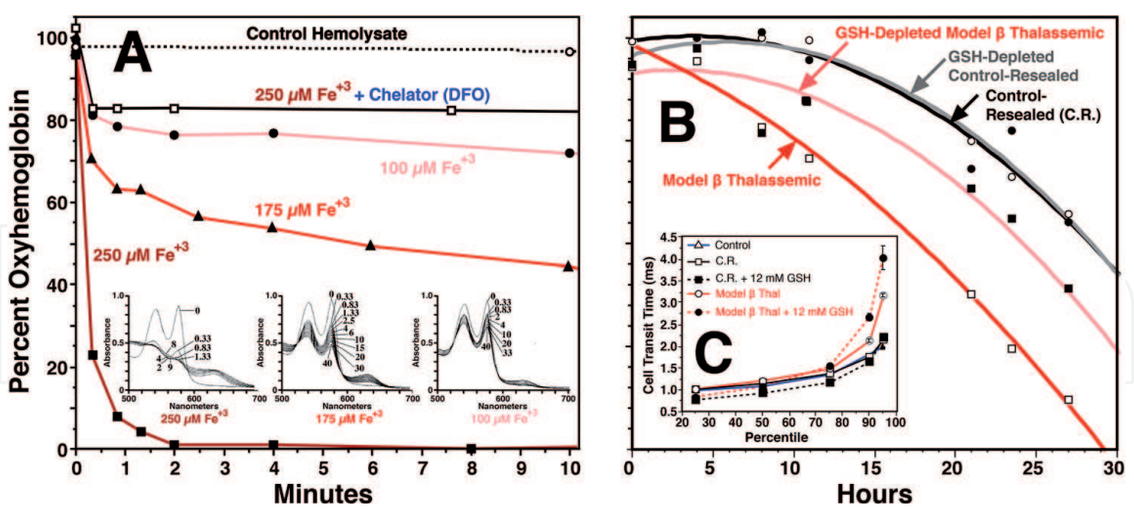

Figure 5.
Role of iron and GSH in the destruction of the β thalassemic RBC. (A) Fe^{+3} is a potent accelerator of hemoglobin oxidation. Shown is the oxyhemoglobin concentration following the addition of 100, 175 and 250 μM Fe^{+3} to a fresh RBC hemolysate. In contrast to the Fe^{+3}-treated samples, no oxidation was observed in the absence of added Fe^{+3} or in the presence of an iron chelator [DFO (deferoxamine) or, not shown, the tripeptide Gly-His-Lys]. Also shown are sequential, hemoglobin scans of the hemolysates treated with Fe^{+3}. The time, in minutes, of the individual scan line following addition of Fe^{+3} are indicated [23, 27]. (B) Depletion of GSH in model β thalassemic RBC inhibits hemoglobin oxidation. Shown is the percent oxyhemoglobin in control-resealed and model β thalassemic cells RBC over 27 h at 37°C. GSH was depleted by treatment with 1-chloro-2.4-dinitrobenzene (CDNB) as previously described [18, 20, 31, 32]. (C) Elevated intracellular GSH levels in model β thalassemic RBC adversely affects cellular deformability as demonstrated by the cell transit analyzer. Less deformable cells take much longer to transit through a pore of known diameter and length. The results shown are the mean ± S.D. of a minimum of four experiments with >2000 RBC measured at each time point. From references: [23, 27].

Population	Hours (37°C)	GSH (μmol/g Hb)	NADPH/$NADP_{total}$ (1.0 = Normal)	Cat. activity (IU/g Hb)
Control RBC	0	5.6 ± 0.4	0.870	147,300 ± 17,000
	20	6.0 ± 0.6	1.063	154,100 ± 14,800
Model β Thal	0	5.2 ± 0.3	0.898	145,600 ± 12,300
	20	2.1 ± 0.0*	0.478*	89,540 ± 8200*

*Modified from [27]. *p < 0.001 from time-matched Control RBC.*

Table 3.
Association between NADPH, GSH and catalase.

(**Table 3**). Catalase is the pre-eminent defense against H_2O_2 within the RBC, making β thalassemic cell particularly increasingly sensitive to H_2O_2 generated via the pathway described in **Figure 4** [31–34, 43]. Following 20 h incubation at 37°C, only 61.5 ± 2.9% of the initial catalase activity remained in the α-hemoglobin chain loaded cells versus 104.6 ± 4.5% in the control RBC. The loss of catalase arose due to the decrease in the NADPH/$NADP_{total}$ ratio as studies have demonstrated that NADPH is essential for maintaining catalase in an enzymatically active state [31–34, 43–47]. Indeed, as noted in **Table 3**, the model β thalassemic erythrocytes exhibit a significant decrease in the NADPH/$NADP_{total}$ ratio similar to that seen in severe G6PD deficiency. Hence, consequent to the oxidation of hemoglobin and the formation of free radicals in the thalassemic RBC, significant metabolic and functional changes are noted in the model β thalassemic RBC that mirror those seen in patient derived samples (**Figure 4** and **Table 2**). It is also important to note that non-oxidative driven damage also occurs. As shown in **Figure 4**, precipitated globin proteins, as well as iron and heme, can alter the intracellular viscosity of the cytoplasm and interact with the cytoskeleton and membrane lipids resulting in mechanical dysfunction. These oxidative and non-oxidative injuries can dramatically affect the function of the RBC.

4. Loss of RBC deformability and vascular survival

From a functional standpoint, perhaps the most important consequence of the oxidative changes to the β thalassemic RBC, as well as other RBC abnormalities, is the loss of cellular deformability [18–27, 29–33, 48–52]. The physiology, fluidics and vascular bed of the circulatory system impart unique rheological stresses on circulating RBC (**Figure 6A**) [53]. These include extreme variations in shear stress and viscosity as well as biomechanical obstacles (e.g., capillaries and splenic filtration). With an average resting cardiac output of approximately 5 L/min, blood flow varies from approximately 40 cm/s in the aorta to 0.03 cm/s in the smallest capillaries [53, 54]. Moreover, blood viscosity (affecting shear stress) is also variable. At high RBC counts and high flow rates, blood is highly viscous while at low RBC counts and low flow rates (capillaries), blood viscosity is greatly reduced. Rheological stress is further exacerbated by the biomechanical stresses induced by the extreme disparity in the size of RBC (~8 μm) to the minimum diameter of the vascular capillary beds (4–5 μm) and splenic interendothelial clefts (0.5–1.0 μm) [55, 56]. Hence, consequent to the shear forces, viscosity and biomechanical stresses placed on blood cells, a key biologic/physiologic requirement of the RBC within the vascular space is rheological deformability. Biomechanically, the intracellular viscosity and membrane rigidity of the RBC are the key factors in imparting their vascular

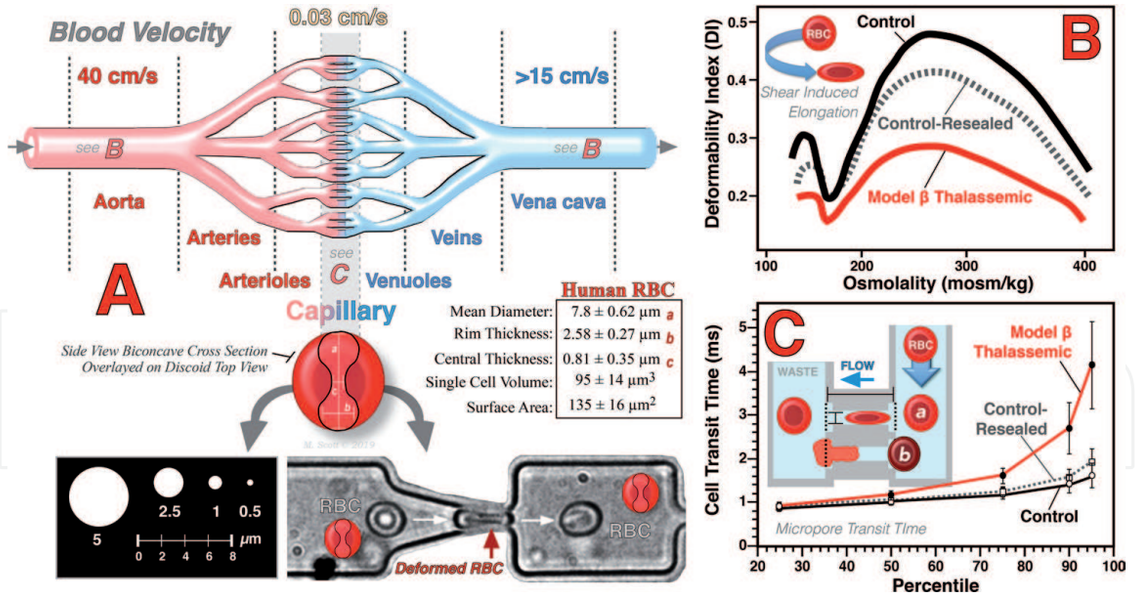

Figure 6.
Vascular deformability of model β thalassemic RBC. (A) The vascular bed is composed of blood vessels of various sizes which create significant disparity in blood (fluid and cellular) velocity consequent to vessel diameter. The fluid flow induces rheological sheer stress while the vessel size creates biomechanical deformation of cellular elements. Shown is an RBC undergoing deformation in a microfluidic channel. (B) Ektacytometric analysis of α-chain loaded RBC demonstrate that these chains dramatically reduce the sheer-induced shape change of the cell [24]. Ektacytometry bests approximates high flow rates. (C) The mean cell transit time (in ms) of model β thalassemic cells was significantly increased, similarly to that observed in patient samples [20]. This microfluidic flow best approximates capillary flow.

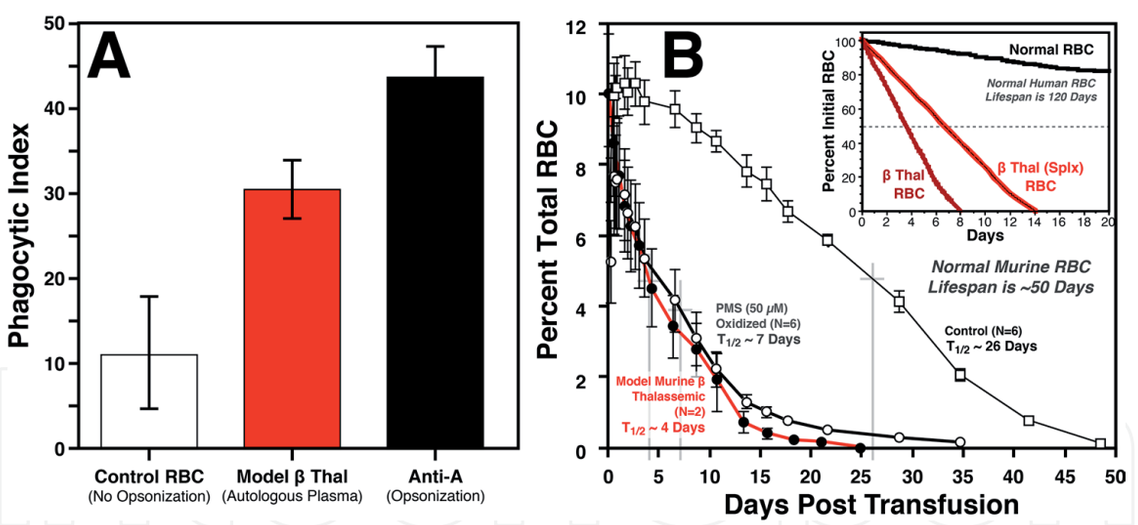

Figure 7.
Model β thalassemic RBC are immunologically recognized and cleared rapidly from the peripheral circulation in vivo. *(A) Blood group A RBC that were loaded with purified α-chains were phagocytosed by human monocytes. Shown for comparison are control cells from the same donor that were either unopsonized or opsonized with an anti-A blood typing antibody. (B) Model β thalassemic mouse RBC were made by the entrapment of purified human α-hemoglobin chains within Normal murine RBC. Oxidatively damaged RBC were made by treating mouse RBC with 50 μM phenazine methosulfate (PMS) for 2.5 h then washing. Both PMS-treatment and the entrapment of α-hemoglobin chains lead to membrane iron deposition and extensive RBC oxidation. The model β thalassemic and PMS-treated RBC have half-lives of 4 and 7 days, respectively in the peripheral circulation versus 26 days for normal murine RBC. Shown is the percent total RBC mass that is PKH-26 labeled. Inset: The peripheral blood lifespan of the human β thalassemic RBC are approximately 6–10 days in a patient with a functioning spleen and 12–16 days in a splenectomized individual. Normal RBC circulates approximately 120 days, data modified from Blendis et al. [69].*

rheological deformability; both of which can be dramatically altered consequent to hemoglobin oxidation, heme release and/or redox-damage to cytoplasmic, cytoskeletal, or membrane components (**Figure 4**) [57–68]. Indeed, as shown in **Figure 6B**,

ektacytometric analysis of the model β thalassemic RBC shows a significant loss of cellular deformability induced by shear stress (e.g., large vessels). Moreover, cell transit analysis of these cells (analogous to capillary deformation) showed a very significant loss of deformability in the model β thalassemic cells as reflected by the very large and significant increase in transit time (**Figure 6C**).

Consequent to the loss of deformability and immune recognition (e.g., Kupffer cells of the liver and, potentially, antibodies), the circulatory survival of β thalassemic RBC is impacted. As demonstrated in **Figure 7A**, model β thalassemic RBC (Blood group A) exhibited enhanced immune recognition and phagocytosis by autologous monocytes when compared to control cells from the same donor. Indeed, the level of phagocytosis was similar to that of the anti-A opsonized positive control RBC. The loss of deformability and enhanced immune recognition both contribute to decreased *in vivo* survival. This was demonstrated using mice transfused with model β thalassemic murine cells (mouse RBC + human α-chains) in which the transfused RBC exhibited a dramatic reduction in the circulatory lifespan (**Figure 7B**). The role of α-chain mediated oxidation was supported by the finding that lightly oxidized (phenazine methosulphate treated; 50 μM) murine cells showed similar circulatory dynamics. These results are comparable to that observed in humans where, consequent to the α-chain driven oxidation, β thalassemic RBC have a very short circulatory lifespan (7–14 days depending on spleen status) compared to the 120 days of a normal RBC.

5. Immunological dysfunction: Effect on antigen presentation

Interestingly, thalassemias have been clinically associated with an increased risk of recurrent bacterial infections [70–87]. This is most evident in under-developed nations where sanitary and medical facilities are most lacking. Despite the clinical evidence of recurrent bacterial infections in thalassemic patients, the biological events underlying this finding are unclear. This confusion arises as a natural consequence of the heterogeneity of the microbial disease itself, the patients age, the state of splenic function, the frequency of transfusion, the degree of similarity between the patient and the blood donor pool, the nutritional status of the patient (e.g., United States versus Thailand) and whether one is looking at humoral or cell-mediated immunity [70–87].

In general, studies on the humoral (i.e., immunoglobulin-based) immunity of thalassemic patients suggest that this arm of the immune system is 'relatively' normal. These studies have indicated normal to elevated levels of IgG, IgA, and IgM but decreased levels of Factor B, C3, and C4 (perhaps due to consumption via oxidatively damaged β thalassemic cells). Reflective of this normality, and consequent to the extensive oxidant injury to the thalassemic cells, circulating immune complexes and an elevated risk of autoimmune hemolytic anemia have been described in β thalassemia intermedia and major patients. Serum fractions from these patients also exhibited increased amounts of C1q-precipitable immune complexes. In contrast, as suggested by the clinically described recurrent bacterial infections, cell-mediated immunity is highly suspect in the thalassemic patient (and sickle cell patients). The few direct studies on cell-mediated immunity in thalassemic patients were, typically, enumeration of the mononuclear cell populations (T cells, B cells, NK cells and monocytes). In general, these studies suggest normal cell numbers but a skewed distribution of the $CD4^+$ to $CD8^+$ T cell ratio. The altered ratio was characterized by a relative depression in $CD4^+$ T cells (i.e., helper T cells) and NK (Natural Killer) cells and a relative rise in $CD8^+$ (cytotoxic and suppressor) T cells that increased linearly with the number of units transfused. However, very

few functional studies have been done in thalassemic patients to answer the question: *Why are β thalassemic patients at risk of recurrent bacterial infections?*

Previous studies have suggested that increased bioavailable iron in transfused patients might facilitate the growth of organisms in which iron is a limiting nutrient (i.e., most bacteria). Other studies have implicated the loss of splenic function. While both of these factors may indeed play important roles in recurrent bacterial infections, they may not offer a complete explanation. In addition to thalassemia, a number of other diseases and trauma scenarios are characterized by recurrent bacterial infections (e.g., malaria and burn injury) suggestive of impaired cell-mediated immunity. Interestingly, a common characteristic of all these conditions is erythrophagocytosis. Previous studies have demonstrated that phagocytic uptake of IgG-coated and oxidatively stressed RBC resulted in a transient depression of further macrophage phagocytosis, decreased respiratory burst (i.e., NADPH-oxidase activity; O_2^- production), and impaired killing of bacteria [88–93]. Interestingly, in the case of *Plasmodium falciparum*-infected RBC, only phagocytosis of mature (trophozoite), but not immature (ring stage), stages had an inhibitory effect on monocyte function. Importantly, a major difference between the mature and immature malarial infected RBC is the presence of malarial pigment (hemozoin), an iron/heme rich degradation product of parasite hemoglobin catabolism. The heme- and iron-rich membranes of the β thalassemic RBC, which we have previously documented [22], may function in a manner analogous to malarial pigment or iron salts and impair cell-mediated immunity—primarily at the level of the APC but potentially extending to the T cell level. Some data from thalassemic patients support the hypothesis for impairment of the T cell response. For example, patients with thalassemia intermedia have been reported to have diminished T cell mitogen responses when their serum iron and ferritin were higher than 200 and 600 μg/dl, respectively [94].

Hence, injury arising from the iron-GSH pathway can result in (any combination of) RBC opsonization by endogenous antibodies, phosphatidylserine (PS) exposure, protein clustering, sublytic levels of complement binding, and/or loss of cellular deformability (**Figure 7**) that leads to the removal of the damaged β thalassemic cells from the circulatory system by components of the mononuclear phagocytic system (MPS). Erythrophagocytosis can occur within the spleen (if present and functioning), liver (Kupffer cells) or the microvasculature itself when

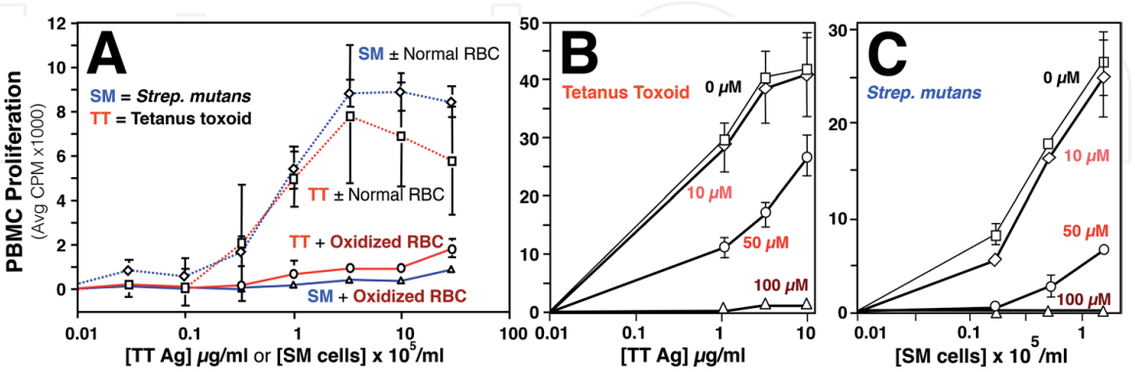

Figure 8.
Antigen processing and presentation is inhibited by oxidized RBC and hemin. (A) Antigen presentation of TT and SM was inhibited by the erythrophagocytosis of oxidized RBC. Normal RBC had no inhibitory effect. The efficacy of antigen processing and/or presentation was assessed by ^{3}H-thymidine incorporation in proliferating T cells. PBMC were resuspended in Aim V media at a final concentration of 2.5 × 10^5 PBMC per 200 μl. Final RBC concentration was 8 × 10^6 per 200 μl. Antigens were diluted in Aim V and added at the indicated concentrations. Results shown are of a representative experiment with quadruplicate samples. (B, C) Heme pretreatment of PBMC (2 h at 37°C) dramatically inhibits the proliferative response to tetanus toxoid (B) and S. mutans *(C). Interestingly, the ability of PBMC to respond to intact bacteria is more significantly blunted than is the response to tetanus toxoid at intermediate hemin concentrations (50 μM). Results shown are of a representative experiment with quadruplicate samples.*

non-deformable RBC are trapped and then cleared by circulating macrophages. Regardless of the location of removal, erythrophagocytosis results in impaired MPS function. As shown in **Figure 8A**, antigen presentation of purified tetanus toxoid (TT; a peptide) or fixed, intact, *S. mutans* (SM; an intact bacteria) by normal human antigen presenting cells (APC; blood monocytes) was dramatically, and differentially, affected by the presence of either control (unoxidized) or oxidized (50 μM PMS as per **Figure 7**) human RBC. As shown, oxidized RBC prevented successful antigen presentation to human T cells while normal RBC showed no detrimental effects. Further experimentation demonstrated that the inhibitory effect was due to heme/iron. As shown in **Figure 8B, C**, direct addition of hemin to the APC impaired successful antigen presentation of both tetanus toxoid and Strep. mutans in a dose dependent manner.

6. Conclusions

β thalassemias arise from a number of underlying genetic defects that interfere with the synthesis of the β hemoglobin chain and the subsequent production of the normal $\alpha_2\beta_2$ hemoglobin tetramer. As a consequence of this decreased/absent β-chain synthesis, unpaired, monomeric, α-hemoglobin chains are produced. While the presence of the highly unstable α-chains mediate the pathophysiology of the RBC, it has been difficult to fully elucidate the mechanisms underlying their destructive processes in human cells. This lack of understanding of the mechanisms of α-chain mediated damage is due, in large part, to the fact that peripheral RBC isolated from β thalassemic individuals are already severely damaged cells (with most being destroyed within the bone marrow). Moreover, severe β thalassemia patients are typically transfused to both correct the severe anemia accompanying the disease and to prevent endogenous erythropoiesis of defective RBC. Hence, murine models of β thalassemia have been developed and extensively studied. However, problems exist with these models (e.g., mouse vs. human α-chains; interaction of human globins with mouse cytoskeletal proteins) and these mice, as in human patients, still suffer from the heterogeneity of RBC changes arising from the different ages of the peripheral blood RBC [11–17].

To better study the *fate of unpaired α-chains* in human RBC, the model β thalassemic cell was developed [18–34]. The entrapment of purified α-hemoglobin chains within normal erythrocytes via osmotic lysis and resealing provides an excellent and reproducible human model for studying the pathologic effects of the unpaired α-chains on the structural and functional characteristics of the RBC. Indeed, as noted in **Table 2**, the α-chain induced structural and functional RBC changes are very similar to those observed in human donor derived β thalassemic RBC. Schematically the pathophysiology of the β thalassemic RBC, and its downstream consequences, as elucidated by the model human β thalassemic RBC, are summarized in **Figure 9**. Importantly, these studies have demonstrated that the unpaired α-chains initiate an *iron, GSH-dependent, self-amplifying and self-propagating reaction* with the subsequent release of even more heme and, eventually, free iron (**Figure 4**). Membrane proteins and reactive thiol groups (not shown) were rapidly decreased in a pattern similar to that observed *in vivo* in β thalassemia [18, 20–23, 25–27]. These oxidative events also result in membrane vesiculation of the thalassemic RBC. One consequence of membrane vesiculation is the preferential loss of phosphatidylinositol (PI) anchored proteins from the RBC. Among these PI-anchored proteins are decay accelerating factor (DAF; CD55) and the membrane inhibitor of reactive lysis (MIRL; CD59) both of which play important roles in preventing complement-mediated binding and lysis. The effects of the

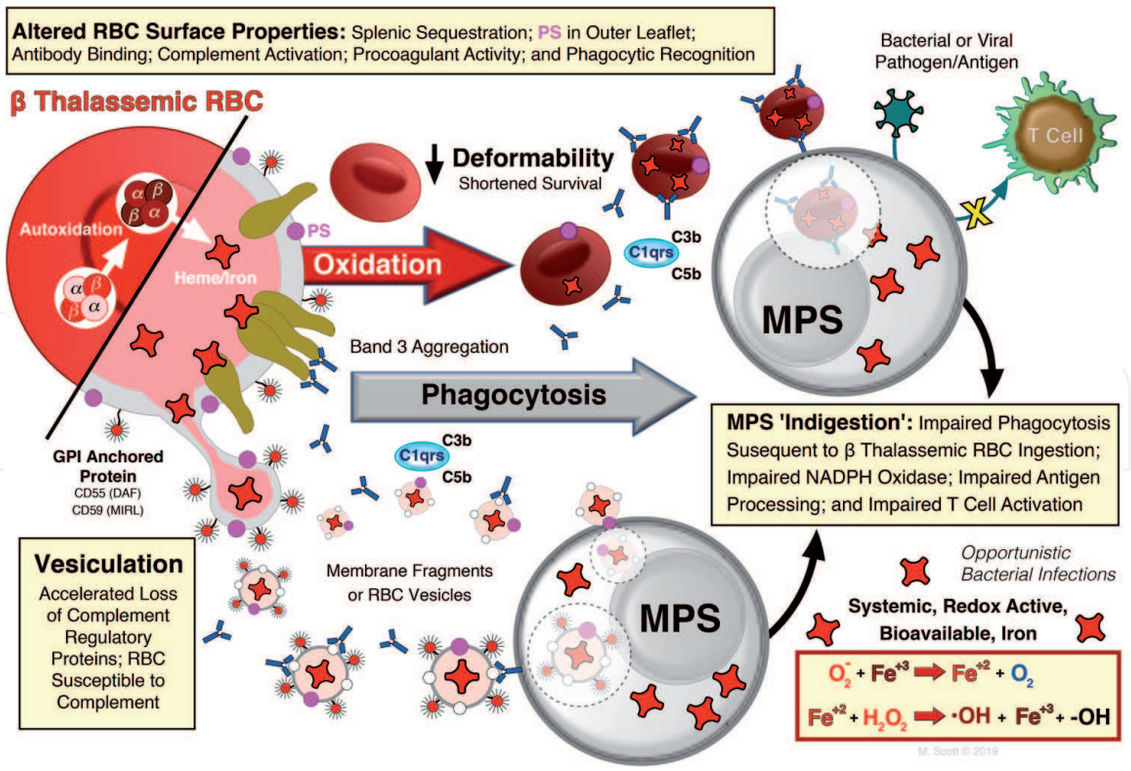

Figure 9.
Schematic representation of the pathophysiology of the β thalassemic RBC and its immunological consequences.

vesiculation-mediated loss of CD55 and CD59 can range from sublytic levels of bound complement enhancing phagocytosis to overt hemolysis. Indeed, a common endpoint for all the α-chain mediated injury is enhanced erythrophagocytosis. As shown, oxidized RBC or the heme from these cells (**Figure 8**) significantly inhibits antigen processing, presentation and T cell proliferation. The systemic importance of this on cell-mediated immunity has not be fully appreciated and may potentially explain the predisposition of thalassemic patients to recurrent bacterial infections.

In sum, these findings show the utility of the model β thalassemic human RBC for investigating the pathophysiology of the unpaired α-chains. Moreover, these cells are easily 'manufactured' from normal donor RBC and may provide an effective means to evaluate therapeutic approaches to ameliorate the damage to the thalassemic cell in β thalassemia intermedia in order to prolong RBC survival and reduce transfusions [23, 25–27, 40].

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Conflict of interest

There are no conflicts of interest.

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