To the Graduate Council:

I am submitting herewith a thesis written by Melissa Overcash entitled "Studies on the Immune System of the Transgenic Sickle Cell Mice, MHOAH-Tg58RuPp." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

Raymond A. Popp, Major Professor

We have read this thesis
and recommend its acceptance:

Salil K. Nijyogi

Michael J. Mircenski

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under the rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Melissa Elizabeth Overcash

Date Jan 6, 1995

Studies on the Immune System of the Transgenic Sickle Cell Mouse, MHOAH-Tg58RuPp

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Melissa Overcash

May 1995

DEDICATION

This thesis is dedicated to the loving, living memory of my Momma,

Elizabeth (Mickey) Lorene Horne Overcash.

ABSTRACT

Research was initiated to investigate several immunological parameters of the transgenic sickle cell mouse strain, MHOAH-Tg58RuPp (Mouse with High Oxygen Affinity Hemoglobin-Transgenic #58 Rubin Popp), in order to advance the use of these mice as a model for human sickle cell disease.

Humans suffering from sickle cell disease have an increased susceptibility to pyogenic bacterial infections which suggests a possible immune dysfunction. A simple procedure, the hemolytic plaque assay was used to compare the number of antibody secreting cells in spleens of MHOAH-Tg58RuPp, MHOAH and C57BL/6J mice. Both primary and secondary responses to sheep red blood cells were evaluated. An elevated number of IgM secreting cells, in primary and secondary responses, was detected in MHOAH-Tg58RuPp mice relative to the two control strains. Fewer plaque-forming cells (PFCs) of the IgG class in the primary response were found in the sickle cell mice than in either C57BL/6J or MHOAH mice. However, in the secondary response the IgG PFCs were much higher in number in the sickle cell mice relative to the controls. Therefore, a study was initiated to delineate the numbers of cells present in various leukocyte subsets in the spleens and peripheral blood of sickle cell and control mice.

Leukocyte subsets were identified by flow cytometric and immunofluorescence methodologies. Antibodies were used to detect cell surface antigens present on the separate leukocyte subsets. Increased numbers of macrophages (MACs) and lymphocytes were found in the spleen of sickle cell mice relative to both C57BL/6J and MHOAH mice. The increase

in the number of lymphocytes was largely due to the increase in B cells and natural killer cells (NK). The T cells in MHOAH-Tg58RuPp were increased but not to the same extent as B cells. The T cell subsets, CD8+ and CD4+ cells, were increased differentially. The helper T cell subset (CD4+) was statistically significantly increased while the cytotoxic T cell subset (CD8+) was not significantly increased. The polymorphonuclear neutrophils (PMNs) were statistically significantly increased. The largest cellular increases in the leukocyte subsets measured occurred in the MAC and NK populations. An increase in MACs could be explained, in part, by the increased use of MACs to remove the misshapen erythrocytes in the sickle cell mice.

Studies involving sickle cell patients use peripheral blood mononuclear cells rather than spleen cells, so a comparative study of peripheral blood and spleen leukocyte subsets of the sickle cell mouse was conducted. Examinations of the peripheral blood subsets revealed results similar to those revealed by the splenic examinations. B cells and T cells (including CD4+ and CD8+) subsets were increased in number. While the NK cells, MACS, and PMNs of the spleen were increased in number compared to controls, there was a decrease in the number of these in peripheral blood subsets. The number of NK cells found in peripheral blood from MHOAH mice is less than half that in sickle cell mice and C57BL/6J mice. Increased numbers of splenic NK cells were found in MHOAH and sickle cell mice (peripheral blood NK cells: MHOAH< sickle cell≤ C57BL/6J, splenic NK cells: C57BL/6J≤ MHOAH<<sickle cell). Numbers of peripheral blood PMNs and MACs are highest in MHOAH, slightly reduced in sickle cell and lowest in C57BL/6J. Numbers of splenic PMNs and MACs subsets are highest in number in sickle cell mice (peripheral blood PMNs and

MACs: C57BL/6J<sickle cell<MHOAH, splenic PMNs MHOAH≤C57BL/6J<<sickle cell and splenic MACs: C57BL/6J< MHOAH<<sickle cell.

Results from these studies suggest that perturbations in the CD8+ T cell subsets in sickle cell mice may be caused by physiological conditions associated with the high oxygen affinity hemoglobin in both MHOAH and sickle cell mice. Perturbations in the B cell and CD4+ subsets appear to be associated with the increase in hematopoiesis and splenic cellularity. Perturbations in the NK, PMN and MAC subsets in the sickle cell mice may result from the production and polymerization of HbS Antilles, red cell sickling, and the pathobiology in several organs of the sickle cell mice. These results show that perturbations exist in MHOAH-Tg58RuPp mice. Nevertheless, the perturbations in the leukocyte subset populations did not cause a deficiency in the immunological functions examined in these transgenic sickle cell mice.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

Introduction

Sickle cell disease is caused by a point mutation, the transversion of adenine to thymine, in the human *beta*-globin gene resulting in an amino acid change of glutamic acid to valine at position six in the hemoglobin (Hb) molecule (Ingram 1957). Although sickled red cells are the grossly visible hallmarks of sickle cell disease, the presence of polymerized deoxy-sickle Hb is the true hallmark of this disease. The solubility of deoxygenated sickle Hb is 1% of the oxygenated form (Perutz and Mitchison 1950). Deoxygenated sickle Hb is only 10% as soluble as deoxygenated normal Hb (Perutz *et al.* 1951). The deoxygenated form of sickle Hb polymerizes and precipitates out of solution leading to the disruption and deformation of the erythrocyte membrane (Frank *et al.* 1985; Serjeant 1992).

A fact little recognized is that every organ system in the body (see Table 1) is adversely affected by the presence of the misshapen and sickled erythrocytes (Serjeant 1992). Effects of sickle cell disease on the cardiovascular system include cardiomegaly, increased stroke volume, increased heart pulse rate, an increased percentage (2.6%-3.5%) of reticulocytes, and a decreased erythrocyte half-life, 8-21 days (normal half-life, 60 days). These effects result from the body's efforts to maintain an adequate supply of oxygen to the tissues. Total Hb is reduced from ~15 g/dl to between 4.5 and 13.0 g/dl. The mean corpuscular volume is elevated which may be due to malfunction of sodium-potassium ion transporters, although this has not been shown conclusively.

Table 1. Effects of Sickle Cell Disease on Human Organ Systems.

Organ System	Pathology	Etiology
CARDIOVASCULAR	cardiomegaly	compensation for lower oxygen
	increased stroke volume	"
	increased heart pulse rate	"
	↓Erythrocyte half-life, 8-21 days	due to destruction of sickled cells
	↑2.6-3.5% reticulocytes	"
	MCV & MCHC elevated	? sodium/potassium ion transporter malfunction
	↓total Hb 4.5-13.0 g/dl	
DIGESTIVE	painful abdominal crisis	referred pain from avascular necrosis of vertebral bone marrow, enlarged mesenteric or retroperitoneal lymph nodes & mesenteric arterial thrombosis
	lowered maximum acid output	?
	malabsorption in small intestine	?
ENDOCRINE	hepatomegagly	?
	decreased levels of plasma cortisol	?
	hyperglycemia	possible primary impairment of insulin secretion
	low testosterone levels	?
	deficiency of growth hormones	?
EXCRETORY	renal hypertrophy, in children	? accommodation for increased cardiac output and renal blood flow
	renal atrophy, in adults	almost complete obliteration of fine vessel system due to sickled cells destruction of vascular system
	hyposthenuria-inability to concentrate urine	
	increased glomerular filtration rate	? increased cardiac output or heart pulse rate
	hematuria	? microinfarction due to sickled cells

(**Source**: Serjeant 1992)

Table 1. (cont.)

Organ System	Pathology	Etiology
IMMUNE	splenomegaly, usually in children can recur splenic atrophy increased susceptibility to bacterial infections defective opsonization abnormalities in alternative & classical complement activation increased number of leukocytes possible abnormal phagocytic function	blockage of splenic blood vessels by sickled erythrocytes " ? ? activation of complement components by membrane of sickled erythrocytes and/or by plasma hemoglobin ? ?
INTEGUMENTARY	leg ulceration	lowered blood supply/infarctions
MUSCULOSKELETAL	bone and joint lesions deformation of skull, long bones, vertebrae, mandible, maxilla, ribs, sternum and hipbone epiphyses dactylitis & osteomyelitis bone age retardation	marrow expansion for increased hematopoiesis avascular necrosis-presumed due to intravascular sickling ?
NERVOUS	sensorineuronal hearing loss hemiplegia, convulsions, coma, stupor and stroke brainstem lesions visual disturbances	auditory nerve compression or ischemic damage to cochlea ? damage to cerebral vessels by blockage of sickled cells " retinal vessel damage
REPRODUCTIVE	priapism delay of puberty gonadal hypofunction	obstruction of venous drainage hematological factors and metabolic rate cardiovascular and endocrine function
RESPIRATORY	acute chest syndrome (chest pain, fever, cough & dyspnoea) lung congestion	infection, acute pulmonary sequestration and infarction secondary to local thrombosis or embolism blockage by sickled cells

(**Source**: Serjeant 1992)

Painful abdominal crisis is classified as a digestive system problem, although some of the pain is referred from avascular necrosis of the vertebral bone marrow and from enlargement of various lymph nodes. Mesenteric arterial thrombosis also contributes to this painful crisis. Patients with sickle cell disease have a lower maximum stomach acid output and malabsorption of nutrients through the small intestines. The basis for such physiological changes in the digestive system has not been determined.

Endocrine system problems include decreased levels of plasma cortisol, hyperglycemia, low testosterone levels, and a growth hormone deficiency. Of these problems only hyperglycemia has a potential explanation, a primary impairment of insulin secretion.

Excretory problems are as follows. Children suffering form sickle cell disease experience renal hypertrophy which may be a compensatory response to increased cardiac output and increased renal bloodflow. Later in life renal atrophy occurs due to the destruction of the fine vessel system. An inability to concentrate urine or hyposthenuria also results from this vessel destruction. Increased glomerular filtration rate has been noted and may be explained by increased cardiac output. Hematuria is probably directly related to the physical presence of sickled erythrocytes causing microinfarctions in the kidneys.

Skin ulcerations of the lower leg and around the heel and ankle are very common to people suffering from sickle cell disease. These ulcerations can be extensive and usually require extended healing times. Lowered blood supply in the region is the most likely explanation for these skin lesions.

Many bones of the musculoskeletal system are adversely affected in sickle cell disease. Expansion of bone marrow for increased erythropoiesis causes deformation of long bones, the skull, vertebrae, ribs, upper and lower jawbones, sternum and hipbone epiphyses. Dactylitis is caused by avascular necrosis in the hands and feet. Osteomyelitis is caused by avascular necrosis in many tissues. Bone age of persons suffering from sickle cell disease is retarded, and so far no explanation has been offered.

Nervous system damage can include loss of hearing and vision. Hearing loss may result from auditory nerve compression caused by bone marrow expansion or ischemic (oxygen deprivation due to obstruction of blood flow) damage to the cochlea. Visual disturbances are a result of retinal vessel damage caused by sickled erythrocytes. Hemiplegia, convulsions, comas, strokes and brainstem lesions are probably caused by damage to cerebral blood vessels.

Effects on the reproductive system are delay of puberty, priapism and gonadal hypofunction. Delay of puberty is caused by a combination of hematological factors and metabolic factors. Priapism is caused by obstruction of the venous drainage. Gonadal hypofunction is a result of vascular damage and an endocrine malfunction of undetermined cause.

The respiratory system problems are manifest as an acute chest syndrome (chest pain, fever, cough and dyspnea). This syndrome has several causes that include infection, acute pulmonary sequestration and infarctions secondary to local thrombosis or embolism. Lung congestion resulting from the presence of sickled erythrocytes and thickening of the

septa (walls) surrounding the aveolar sacs also occur leading to a reduction in functional lung capacity.

The immune system is also affected in sickle cell disease. Several studies have indicated an impaired ability of the immune system to respond to pyogenic bacterial infections (Robinson and Watson 1966; Barrett-Connor 1971; Buchanan *et al.* 1983). Reports by Bjornson and Lobel (1986, 1987) and Bjornson *et al.* (1991) suggest that the increased susceptibility to bacterial infection may be related to a deficiency of immunoglobulin molecules with specificity to the lipopolysaccharide components of bacterial cell walls. Plaque-forming cells capable of producing antibody to pokeweed mitogen are present in higher numbers in asymptomatic sickle cell patients than in control subjects (Venkataraman and Westerman 1985). When these patients became symptomatic, *i.e.*, experienced a period of crisis, the number of PFCs responding to pokeweed mitogen dropped significantly. Studies also revealed an overall increase in the number of lymphocytes in sickle cell patients (Kaplan *et al.* 1984; Ballester *et al.* 1986; Powars and Wong 1993). Ades *et al.* (1980) reported a decrease of at least 50% in T cells in sickle cell patients. Venkataraman and Westerman (1985) repeated the T cell analyses using both an E-rosette formation technique and a fluorescently labeled monoclonal antibody technique. The number of T cells enumerated by the E-rosette formation technique was significantly lower than that enumerated by analysis with the monoclonal antibody CD3 which recognizes an intergral part of the T-cell receptor. Results of this comparative experiment may explain the discrepancy between the report by Ades *et al.* (1980) and the reports by Kaplan *et al.* (1984), Ballester *et al.* (1986) and

Powars and Wong (1993). Kaplan *et al.* (1984) and Ballester *et al.* (1985) reported an increase in the number of CD8+ cells but not of CD4+ cells in sickle cell patients. Powars and Wong (1993) reported an increase in both CD4+ and CD8+ subsets. Kaplan *et al.* (1984) and Ballester *et al.* (1985) reported a decrease in the CD4+/CD8+ ratio, a parameter which can be indicative of certain diseases (*e.g.*, AIDS).

Several mouse models exist for sickle cell disease (see reviews of Fabry 1993 and Nagel 1994). Characteristics of the mouse models so far generated are listed in Table 2.

Ryan *et al.* (1990) created a transgenic mouse line containing two separate gene constructs. These constructs contain the human β^{Sickle} and $\alpha 1$-globin genes under the control of the erythroid-specific β-locus control region. The β-locus control region contains five DNase I hypersensitive sites that normally control expression of the β-globin genes. The control region is also able to control correct *in vivo* expression of human α-globin genes in transgenic mice. To enrich for human sickle Hb these transgenic mice were bred to β-thalassemic mice carrying a deletion in the β-globin major gene. Red cells of mice heterozygous for both the mouse β-globin deletion and the human transgenes contain ~50% human sickle Hb; they are slightly anemic, have elevated reticulocyte counts, have low total Hb and display splenomegaly. At ambient conditions less than 1% of the erythrocytes sickle, but at hypoxic conditions greater than 90% of the erythrocytes sickle. No other organ pathologies were mentioned for this mouse model (Ryan *et al.* 1990).

Table 2. Mouse Models of Sickle Cell Disease.

	Control	Group		Group	Group		Group		Group	Group
Attribute	C57BL/6J	Ryan #1	Ryan #2	Greaves	Trudel #1	Trudel #2	Fabry #1	Fabry #2	Rubin	Popp
Thalassemia	_	MD	MDD	_	_	MD	MDD	AD, MDD	MDD	_
% Hb S	_	50	50	83	19[§]	26[§]	41.9	40.6	8.3[¶]	30[¶]
Hematocrit	45-47%	45.0%	_	49%	44%	39.4%	47%	45.9%	45.9%	48.5%
Hb g/dl	14-16	13.5	_	14.4	16.5	15.2	17.4	17.3	_	15.1
P_{50} mm Hg	40-42	_	_	_	44.6	44.8	33.5	37.4	_	33
Reticulocytes	2-4%	7.4%	_	4.4%	3.6%	6.2%	6.7%	10.3%	2.9%	15.6%
% ISC	_	2.5	_	0.1	_	5-15	2.4	1.4	<1	+
MCV fl	45.4	_	_	47	40.7	35.4	43.0	41.9	44.4	46.4
MCH pg	14-15	_	_	13.8	15.1	13.6	14.1	14.3	_	15.0
MCHC g/dl	33-34	_	_	29.4	37.3	38.6	35.1	36.3	33.5	32.2
Enlarged spl	_	+	_	_	+	+	+	+	_	+
Priapism	_	_	_	_	_	+	_	+	_	_
Ulcerations	_	_	_	_	_	_	_	_	_	_
Lung congestion	_	_	_	_	_	+	+	+	_	+

¶-β^{S4D}($\beta^{S\text{-}S\ Antilles\text{-}D\ Punjab}$, β6Glu→Val, β23Glu→Ile, β121Glu→Gln), §-$\beta^{S\ Antilles}$ (β6Glu→Val, β23Glu→Ile), MD-heterozygous β-major deletion, MDD-homozygous β-major deletion, AD-heterozygous α-globin deletion, Hb S-Hb S, MCV-mean corpuscular volume, MCH-mean corpuscular Hb and MCHC-mean corpuscular Hb concentration, ISCs-irreversibly sickled cells

(**Sources**: Nagel 1994; Trudel *et al.* 1994; Fabry 1993; Popp *et al.* 1993; Trudel *et al.*1991; Fabry *et al.* 1992a and b, Greaves *et al.* 1990 and Ryan *et al.* 1990).

Another transgenic mouse was created by Greaves *et al.* (1990) containing a construct of two human $\alpha 1$-globin genes and one human β^{Sickle}-globin gene under the control of four of the DNase I hypersensitive sites of the β-locus control region. Only one transgenic mouse expressed significant amounts of human sickle Hb (83% of total Hb). This mouse had a normal hematocrit, 4.4% reticulocytes, normal total Hb, slightly elevated mean corpuscular volume (MCV), normal mean corpuscular Hb (MCH), and a low mean corpuscular Hb concentration (MCHC). No obvious symptoms of sickle cell disease were observed. According to Fabry (1993) and Nagel (1994) this mouse was not propagated.

Trudel *et al.* (1991) made two constructs both under the control of four of the DNase I hypersensitive sites of the β-locus control region, to generate a transgenic line of mice that expressed a composite gene comprised of portions of the β-globin genes coding for the β^{S}, $\beta^{SAntilles}$ and the $\beta^{D\text{-}Punjab}$ mutations and the human $\alpha 2$-globin gene. The composite gene is called Hb^{SAD}, and the Hb produced is called Hb^{SAD}. This transgenic mouse produced erythrocytes containing 19% Hb^{SAD}, and the mice exhibited a slightly reduced hematocrit, normal total Hb, 3.6% reticulocytes, a low MCV, normal MCH, a slightly elevated MCHC, and splenomegaly. The P_{50} for the Hb of this mouse is 44.6 mm Hg. This mouse was mated to β-thalassemic mice to produce mice heterozygous for the Hb^{SAD} gene and the β-major deletion. Erythrocytes from Hb^{SAD}/β-thalassemic heterozygotes contain 26% Hb^{SAD}. The hematocrit was lower in these heterozygous mice than in the proband transgenic mouse. Total Hb and MCH values were within normal ranges. The MCV was much lower than normal, and the MCHC was much higher than

normal. The percentage of reticulocytes rose to 6.2% and the irreversibly sickled cell (ISC) counts were between 5% and 15%. The erythrocyte turnover was higher in transgenic Hb^{SAD} than in normal mice suggestive of a high incidence of destruction. These mice also have enlarged spleens. Follow-up studies on this sickle cell mouse model have revealed a very extensive pathology (Trudel *et al.* 1994). Microvascular occlusions occur in the lung, liver, kidneys, bone marrow and spleen, and skin ulcerations were abnormally prevalent (16%, n=125). Priapism and penile hemorrhage have also been noted. Thrombosis in the lungs and renal hypertrophy also occur in these animals. These mice have a shortened lifespan, 15.4 ± 7.3 months as compared to 28 months for CBA/J and 23 months for C57BL/6J control mice (Trudel *et al.* 1994).

Fabry *et al.* (1992) produced a transgenic line of mice with two cointegrated transgenes, the human β^S- and $\alpha 2$-globin genes under control of four of the DNase I hypersensitive sites of the β-locus control region. These transgenic mice were bred to α- and β-thalassemic mice to produce two strains of mice. Both strains were homozygous for the β-major deletion. One strain was additionally heterozygous for an α-globin deletion. Both strains exhibited normal MCH, normal hematocrits, slightly elevated total Hb, an increased number of reticulocytes, a small number of ISCs, increased MCH, and a slightly lower than normal MCV. Spleens, lungs and kidneys from these animals were larger than in normal control animals. Stainable iron was abundant in the spleen, which suggests that the level of erythrocyte destruction is high. The glomerular filtration rate is increased by 25% in these mice.

Rubin *et al.* (1991) used the $\beta^{SAntilles}$- and $\alpha 2$-globin genes under control of the DNase 1 hypersensitive site II to create a transgenic mouse. Hemoglobin $\beta^{SAntilles}$ is coded for by a β-globin allele containing both the β6Glu→Val and the β23Val→Ile mutations. The latter amino acid replacement makes the Hb S Antilles less soluble and have a lower oxygen affinity (P_{50}=~40 mm Hg) than Hb S (P_{50}= ~26 mm Hg). The lower solubility reduces the amount of deoxysickle Hb required to initiate nucleation and polymerization of sickle cell Hb and red cell sickling. In mixtures of Hb S (P_{50}= ~26 mm Hg) and normal mouse Hb (P_{50} =~ 40 mm Hg) initial deoxygenation of normal mouse Hb is favored as erythrocytes pass through small capillaries of the mouse. Since the lowered oxygen affinity of Hb S Antilles is comparable to the oxygen affinity of normal mouse Hb, both hemoglobins are deoxygenated to the same extent within erythrocytes. By using the $\beta^{SAntilles}$-globin gene a transgenic mouse with a more severe pathology may be produced. These transgenic mice were then bred to β-thalassemic mice to reduce the amount of normal mouse Hb synthesized. Reduction of the normal mouse Hb should also favor the deoxygenation of the sickle Hb increasing the chance of producing sickled erythrocytes. Erythrocytes from these mice possess 8.3% Hb S Antilles. No organ pathology was reported, and these mice had normal hemtocrits, 2.9% reticulocytes, less than 1% ISCs, a slightly lower MCV, and a normal MCHC.

MHOAH-Tg58RuPp (Mouse with High Oxygen Affinity Hemoglobin-Transgenic #58 Rubin Popp) was developed by Popp *et al.* (1993) by breeding the transgenic $\beta^{SAntilles}$ mouse developed by Rubin *et al.* (1991) onto the genetic background of the mouse strain, MHOAH (Mouse with High Oxygen Affinity Hemoglobin). MHOAH mice were bred

from two mice, each one possessing a separate ethylnitrosourea induced germline mutation. One mutation in the α-globin gene codes for a α89 His→Leu replacement (Popp *et al.* 1983). The other mutation in the β-globin gene codes for a β59 Lys→Ile replacement (Lewis *et al.* 1985). These mutant alleles have been designated Hba^{g2} (Popp *et al.* 1983) and Hbb^{s2} (Lewis *et al.* 1985), respectively. Mice doubly homozygous for Hba^{g2} and Hbb^{s2} synthesize hemoglobins with high oxygen affinities, hence the name MHOAH. The hemoglobin of MHOAH mice has a P_{50}=24.5 mm Hg (D'Surney and Popp 1992). Human $\beta^{SAntilles}$ and normal mouse hemoglobins have comparable oxygen affinites, P_{50}= ~40 mm Hg (Popp *et al.* 1992; Serjeant 1992, Figure 1). The erythrocytes of MHOAH-Tg58RuPp sickle cell mice contain ~30% human α and $\beta^{SAntilles}$ globins, ~50% mutant mouse α and β globins and ~20% normal mouse α- and β-globins (Popp *et al.* 1993). In erythrocytes of MHOAH-Tg58RuPp mice which contain mixtures of Hb S Antilles (P_{50} =~40 mmHg) and MHOAH Hb (P_{50}=24.5 mm Hg), the initial deoxygenation of the Hb S Antilles is favored as erythrocytes pass through small capillaries of the mouse. This initial nucleation and polymerization of Hb S Antilles causes red cell sickling in MHOAH-Tg58 RuPp mice raised in ambient conditions. These mice exhibit several clinical and pathological symptoms commonly observed in sickle cell patients. These traits include ~15% peripheral blood reticulocytes, many misshapen and dense red cells and ISCs (Popp *et al.* 1993). Several kinds of tissue pathologies associated with human sickle cell disaese are also present. These include splenomegaly, stainable iron in the spleen, liver and kidneys, and congestion of sickled cells in thekidneys and lungs, thickening of the

Figure 1. Oxygen Association/Dissociation Curves.

Figure 1 shows the oxygen association/dissociation curves for mouse high oxygen affinity (MHOAH) hemoglobin, 1; for normal human hemoglobin, 2; for MHOAH-Tg58RuPp hemoglobin, 3; and for normal mouse hemoglobin, 4.

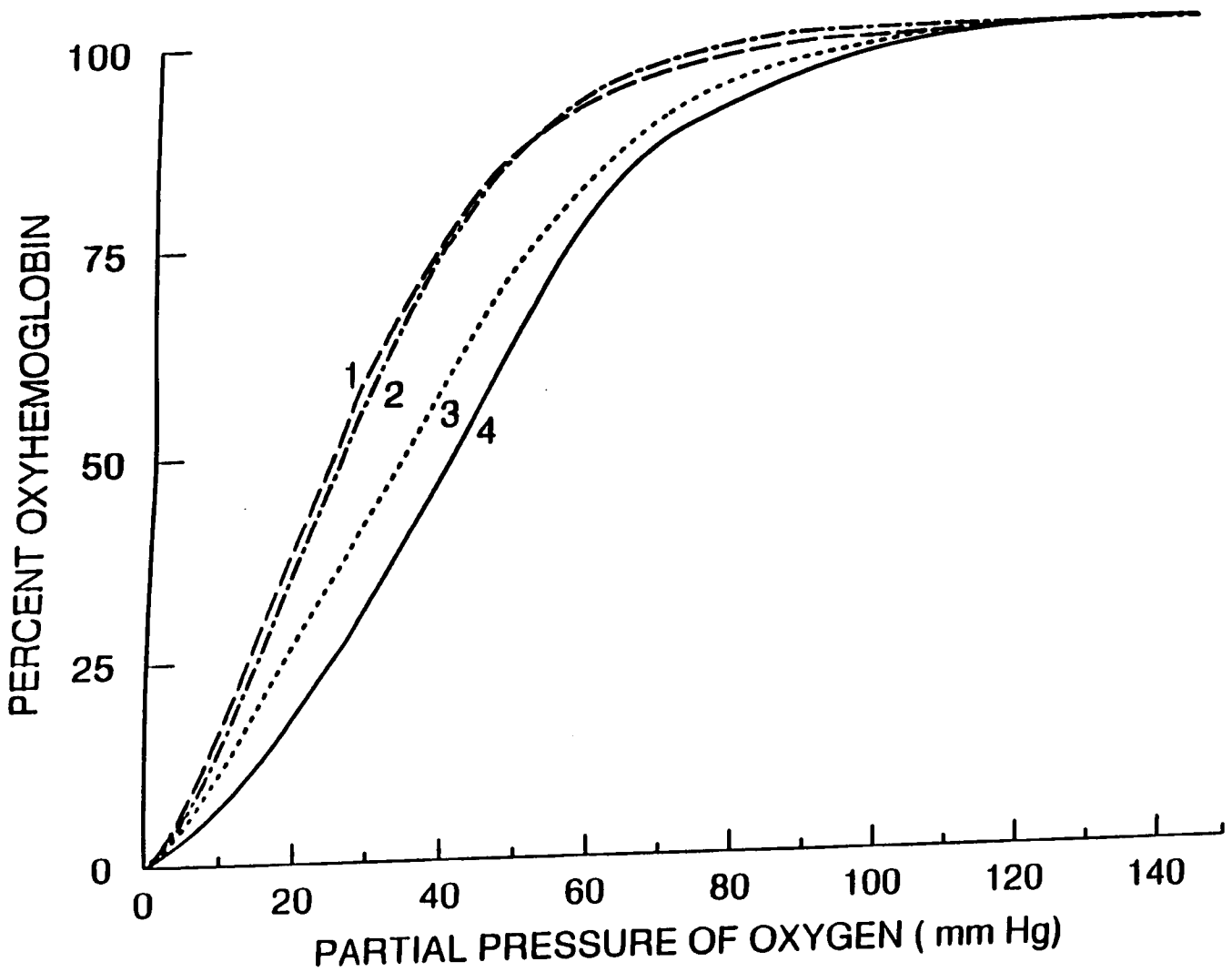


Figure 1. Oxygen Association/Dissociation Curves.

walls surrounding the aveolar sacs and vascular occlusions in the small vessels of the liver, glomeruli and retinas (Popp *et al.* 1993).

Misshapen erythrocytes cause several pathophysiological effects. Erythrocytes of MHOAH-Tg58RuPp sickle cell mice have a reduced half-life, 15 days, as compared to the 21 day half-life of C57BL/6J and MHOAH erythrocytes (Popp *et al.* 1993). The increased erythrocyte destruction and iron-overload in sickle cell mice are pathologies similar to those found in β-thalassemic mice (Popp *et al.* 1990a). Thalassemic mice are immunodeficient (Popp *et al.* 1990b). With the knowledge that humans with sickle cell disease are more susceptible to certain bacterial infections and knowledge of similar pathologies in sickle cell and immunodeficient β-thalassemic mice, a series of experiments was designed to determine if the MHOAH-Tg58RuPp sickle cell mice are immunodeficient and to characterize cellular perturbations that would be expected.

CHAPTER 2

Materials and Methods

Mice

Mice transgenic for human *alpha*-2 ($\alpha 2$) and *beta*-$S^{Antilles}$ ($\beta^{SAntilles}$) genes (MHOAH-Tg58RuPp), murine high-oxygen-affinity-hemoglobin (MHOAH) mice, and C57BL/6J mice were raised by Dr. Raymond A. Popp in the Biology Division at the Oak Ridge National Laboratory following AALAC guidelines.

IgM Hemolytic Plaque Assay

Prior to the hemolytic plaque assay several materials have to be prepared. To prepare the SRBCs (Colorado Serum Company, Denver, CO) they were washed as follows. The SRBCs were removed from the shipping vial by needle and syringe and were placed in a 15 ml screw cap polypropylene tube. A balanced salt solution (BSS: 5.5 M dextrose, 0.3 M KH_2PO_4, 1.3 mM $Na_2HPO_4 \cdot H_2O$, 0.05% phenol red, 1.3 mM $CaCl_2 \cdot H_2O$, 5.3 mM KCl, 1.1 mM $MgCl_2$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.17 M NaCl) was added to fill the tube which was then inverted several times to mix. The SRBCs were centrifuged for 10 minutes at 1000 RPM (224 x g). The liquid was carefully aspirated. The cells were resuspended by flicking the tube several times, BSS was added to 15 ml and the wash steps were repeated twice more.

To prepare the guinea pig complement (GIBCO, Grand Island, New York) a volume of SRBCs, which was ⅓ to ½ of the volume of complement to be absorbed, was washed three times in large volumes of BSS by centrifugation at 500 x g for 15 minutes at 4°C. After the last wash, all of the BSS was removed, and the complement to be absorbed was poured over the packed SRBCs. The cells were resuspended by flicking or inverting the tube several times and incubated on ice for 20 minutes, being sure to invert the tube occasionally to keep the cells in suspension. The cells were pelleted by centrifugation for 15 minutes at 400 x g and 4°C. The absorbed complement with all SRBCs removed was transferred in 2-3 ml aliquots into separate containers and stored at -70°C.

To prepare the agarose-coated slides a solution of 0.1% agarose was prepared by dissolving the agarose (BRL, Gaithersburg, MD) in distilled water (dH_2O). This mixture was rapidly brought to a rolling boil. A fine bristle paintbrush was used to apply the agarose solution to frosted-end slides, which were taped (by the frosted end) to a flat level surface. After the water evaporated from the slides, a wax marking pencil was used to mark the boundary between the frosted end and the agarose coated end.

C57BL/6J, MHOAH and MHOAH-Tg58RuPp sickle cell mice were injected intravenously (using a 27-gauge needle) with 500 µl of a freshly washed 2% SRBC suspension in BSS. Three to six days after receiving an injection of SRBCs, the mice were sacrificed by cervical dislocation. The spleens were removed and placed into 15 ml snap cap round bottom, polypropylene tubes on ice containing 2 ml BSS. Teflon pestles were used to disperse the cells of the splenic tissue into single cell suspensions. Ten ml of BSS

was added to the tube which was inverted several times and then centrifuged for 10 minutes at 1000 RPM (224 x g). The liquid was decanted and the cell pellet was resuspended to 10 ml with BSS. Serial dilutions of these suspensions were used to determine the number of cells secreting immunoglobulin M (IgM) antibody to SRBCs. Dilutions of spleen cells were made according to previously determined values (a volume that would insure between 20 and 200 plaques per slide). Anti-SRBC IgM, produced by immunized plasma cells, in the presence of complement cause lysis of SRBCs in the support agarose surrounding the plasma cells. Two or three different volumes (100 µl, 50 µl and 25 µl) of a diluted splenic suspension were mixed with 500 µl of the 0.6% agarose (BRL, Gaithersburg, MD) dissolved in BSS. Just before the addition of the splenocytes, 50 µl of a freshly washed 6.6% suspension (1:15 of packed red cell volume) of SRBCs was added to the 500 µl of 0.6% agarose in the test tubes. The samples of spleen cells, SRBCs, and agarose were then vortexed to mix and poured onto prepared agarose-coated glass microscope slides. After the mixtures were poured aliquots of the remaining spleen cell suspensions were stained with 0.1% gentian violet in 0.01% acetic acid $_{\text{aqueous}}$ and the number of spleen cells in each suspension was counted on a hemocytometer. Triplicate slides were poured for each spleen cell dilution. The slides were then placed upside down in a special tray (which contains raised ledges on which the very ends of the poured slides rest). A cold solution of 1:20 BSS: preabsorbed guinea pig complement, was flooded into the tray using a 10 ml pipet. Bubbles that can form under the slides were removed. The preparations of slides and complement were held for three hours at 37°C in a humidified incubator. After the incubation period the slides were rinsed in 0.15 M saline

to remove the complement. Plaques (clear circles appearing on the slide where SRBC coated with antibodies were lysed by complement) were counted with the aid of a magnifying glass and indirect light and recorded. Plaques that were very large or displayed uneven boundaries (possibly indicating multiple antibody secreting cells) were not counted. If a plaque was questionable a microscopic examination was conducted to determine if a single cell was at the center of the plaque.

IgG Hemolytic Plaque Assay

Mice to be tested for secondary hemolytic plaques were injected ten days following the first injection and sacrificed on the fifth day post secondary injection. The procedure for IgM (direct plaques) was followed for the determination of IgG (indirect) plaques up through the point of pouring the agarose-SRBCs-spleen cell mixture (Henry 1980). IgG does not fix complement as well as IgM. Therefore to detect IgG plaques an exogenous antibody specific to mouse IgG molecules and capable of fixing complement is added. This exogenous antibody will bind to IgG secreted by the spleen cells. If the IgG is bound to SRBCs the exogenous complement fixing antibody will fix the complement causing the SRBCs to lyse producing a plaque. Since the plaques are produced indirectly through the exogenous antibody the assay for IgG is called an indirect assay. The number of plaques produced by this method includes IgM plaques as well as IgG plaques. So, to generate the number of IgG plaques the number of IgM plaques must be substracted from the total plaques. The exogenous antibody will inhibit the formation of IgM plaques to some extent so the percent of IgM plaque inhibition must be determined (Henry 1980). In

order to determine the percent inhibition a plaque assay is performed when only IgM plaques are being produced. This happens on day three following injection of SRBCs. Two sets of assays are performed on day 3 post injection. One set includes the exogenous antibody and one set does not. The percent difference in the number of plaques produced in each set is the percent IgM plaque inhibition caused by the exogenous antibody. Two sets of slides each at multiple dilutions were poured for the IgG PFC assay, one set for a direct plaque assay and one for an indirect plaque assay. After the slides were poured for the direct plaque sets they were flooded with a 1:20 dilution of preabsorbed guinea pig complement (volume of complement needed was based on a volume of approximately 1.5 ml per slide poured). The slides for the indirect plaque set were flooded with a previously titrated rabbit anti-mouse Ig (1:200, CedarLane Laboratories Limited, Ontario, Canada) and complement (1:20) in 1X BSS and the preparation was incubated for three hours at 37°C in a humidified incubator without CO_2. The solution of complement or anti-mouse Ig and complement was aspirated and the trays were flooded with 0.15 M saline to dilute the remaining complement and halt lysis. The plaques were counted with the aid of a magnifying glass and an indirect light and recorded.

Flow Cytometric Analysis of Splenic Leukocyte Subsets

Mice were sacrificed and the spleens were removed. The splenic tissue was dispersed to generate single cell suspensions in S-PBS (Mg^{++}/Ca^{++}-free phosphate buffered saline, PBS: 0.15 M NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$-KH_2PO_4). . The cells were washed 1X (Samples were centrifuged for 10 min at 1000 RPM; the supernate

decanted, and the cells were resuspended). This step and all steps working with cells were carried out on ice or at 4°C. An aliquot of cells was resuspended in 2-5 ml of S-PBS^s (the superscript defines supplemented and refers to the BSA and the sodium azide) which contained added 0.1% bovine serum albumin fraction V (Sigma Chemical Co., St. Louis, MO) and 0.1% sodium azide (Fisher Scientific Co., Fair Lawn, NJ) to prevent capping of the bound antibodies on the cell membrane, diluted in 0.01% acetic acid $_{aqueous}$, 0.1% gentian violet, and counted by the use of a hemocytometer. The concentration of cells was then adjusted to 1 x 10^7 cells per ml. An aliquot of 0.1 ml (1 x 10^6 cells) was used for antibody staining. Antibody to Fc_γ receptors was added to some cell aliquots to block the binding of labeled antibody to cells through the Fc portion (constant region of classes of antibodies) of some of the antibodies used. Antibody was added to the cells in specified amounts and incubated for 30 minutes on ice. Two ml of S-PBS^s was added to the cells, and they were washed 2X (as above). Finally, the cells were resuspended in 1 ml of S-PBS^s. Five to 10 minutes before analysis, 10 µl of a propidium iodide (PI, 1.0 mg/ml) solution was added to the cell suspension to identify dead cell (*i.e.*, cells with compromised plasma membranes are unable to exclude PI from their cytoplasm). The cells were analyzed by a Becton Dickinson FACStar^{PLUS} flow cytometer (argon laser set at 488 nm). Fluorescent intensity, forward scatter and side scatter data were acquired from 10,000-20,000 events. Collected data were then analyzed using the Multi2D™ analysis software. The PI positive cells were excluded from final analysis by software gating.

Monoclonal Antibodies

The following monoclonal antibodies were used in the course of this research: αThy-1.2 [clone: 5a-8, mouse IgG_{2b} (Ledbetter *et al.* 1980; van Ewijk *et al.* 1981), Caltag Laboratories, San Francisco, CA], CD4 (L3/T4) (clone: YTS 191.1, rat IgG_{2b}, Caltag Laboratories, San Francisco, CA) or CD4 (clone: RM4-4, rat IgG_{2b}, PharMingen, San Diego, CA), CD8a (Ly-2) (clone: YTS 169.4, rat IgG_{2b}, Caltag Laboratories, San Francisco, CA), CD45R [B220/Ly-5, clone: RA3-8B2, rat IgG_{2a} (Coffman 1982; Coffman and Weissman 1981), PharMingen, San Diego, CA)], F4/80, α-mouse macrophage/monocyte, [clone: F4/80, rat IgG_{2b} (Austyn and Gordon 1981; Lee *et al.* 1985), Biosource International, Camarillo, CA], NK 1.1 Biotin conjugated [clone: PK 136, mouse IgG_{2a} (Koo and Peppard 1984), PharMingen, San Diego, CA], CD32 [α-FcγII Receptor, clone: 2.4G2, Rat IgG_1 (Titus *et al.* 1984), PharMingen, San Diego, CA]. For the detection of cells by the NK 1.1 antibody, a streptavidin-conjugated R-phycoerythrin was used (PharMingen, San Diego, CA).

CHAPTER 3

Results

Hemolytic Plaque Assay

Data on primary and secondary IgM PFCs in the spleen (Table 3) show that sickle cell mice have a larger number of primary IgM PFCs (2.94×10^5) than either MHOAH (1.42×10^5) or C57BL/6J (1.17×10^5) mice. Sickle cell mice also have more secondary IgM PFCs (1.08×10^5) than MHOAH (0.282×10^5) or C57BL/6J (0.146×10^5) mice. Results on primary and secondary IgG PFCs (Table 4) in the spleen show that sickle cell mice have fewer primary IgG PFCs (0.42×10^5) than either MHOAH (1.2×10^5) or C57BL/6J (1.6×10^5) mice. However, sickle cell mice have more secondary IgG PFCs (4.9×10^5) than either MHOAH (1.4×10^5) or C57B/6J (1.25×10^5) mice. SRBC-immunized and non-immunized sickle cell mice have an increased splenic cellularity compared to MHOAH and C57BL/6J mice (see Figure 2). Immunized sickle cell mice have $510 \times 10^6 \pm 23.8$ cells/spleen, MHOAH have $254 \times 10^6 \pm 9.17$ cells/spleen and C57BL/6J mice have $211 \times 10^6 \pm 12.6$ cells/spleen. The increase in splenic cellularity between the immunized sickle cell mice and the immunized control mice is approximately two-fold. Non-immunized sickle cell mice have $374 \times 10^6 \pm 41.0$ cells/spleen, MHOAH mice have $122 \times 10^6 \pm 4.4$ cells/spleen and C57BL/6J mice have $110 \times 10^6 \pm 5.4$ cells/spleen. The increase in splenic cellularity between non-immunized sickle cell mice and the non-immunized control mice is approximately three-fold.

Table 3. Primary and Secondary IgM Plaque-forming Cells per Spleen (x 10^5).

	Strain		
	C57BL/6J	MHOAH	MHOAH-Tg58
1°IgM PFC	1.17 ± 0.15^a	1.42 ± 0.14^b	2.94 ± 0.38^c
2°IgM PFC	0.14 ± 0.02^g	0.282 ± 0.08^d	1.08 ± 0.35^g
Cells/Spleen (x10^6)§	211 ± 12.6^b	254 ± 9.17^e	510 ± 23.8^f

Data are presented as the mean ± SEM. § $p < 0.001$; 1° PFC: MHOAH vs. Tg58 $0.002 < p < 0.005$, C57BL/6J vs. Tg58 $0.005 < p < 0.01$; 2° PFC: MHOAH vs. Tg58 $0.2 < p < 0.1$, C57BL/6J vs. Tg58 $.05 < p < 0.02$. a, n = 9; b, n = 13, c, n = 22; d, n = 4;e, n = 19; f, n = 26, g, n = 7.

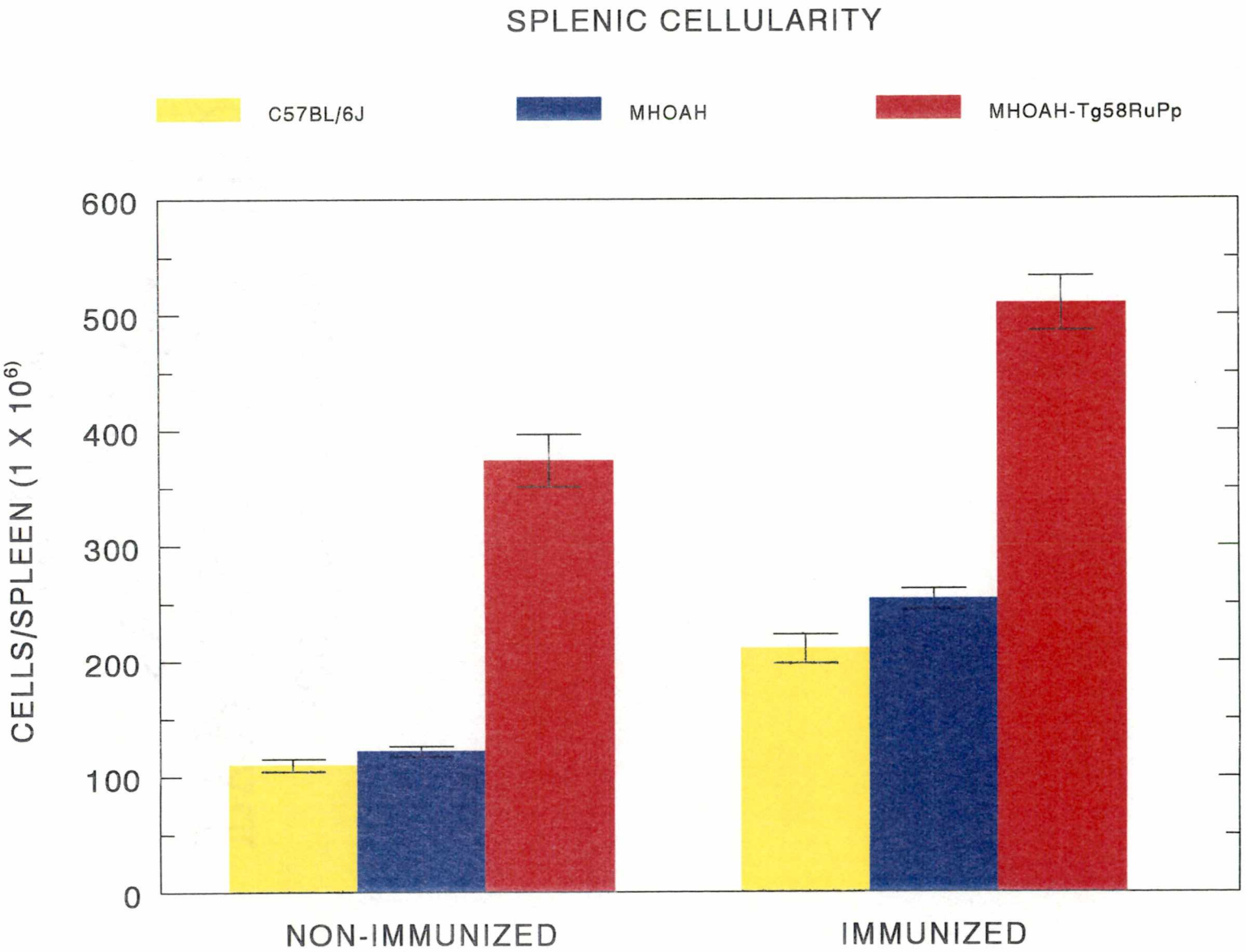
Table 4. Primary and Secondary IgG Plaque-forming Cells per Spleen (x 10^5).

	Strain		
	C57BL/6J	MHOAH	MHOAH-Tg58
1° PFC IgG	1.6 (0.19-3.1)	1.2 (0.37-2.2)	0.42 (0.27-0.56)
2° PFC IgG	1.25 (0.33-2.7)	1.4 (0.78-2.5)	4.9 (2.7-8.97)
Cells/Spleen (x10^6)	211 (126-280)	254 (180-321)	510 (290-708)

Data are presented as the mean with the range in parentheses. 1°IgG: C57BL/6J, n = 2, MHOAH, n = 5 and Tg58, n = 2; 2°IgG: C57BL/6J, n = 7, MHOAH, n = 4 and Tg58, n = 6.

Figure 2. Splenic Cellularity.

Splenic cellularity of SRBC immunized and non-immunized C57BL/6J (yellow), MHOAH (blue), and MHOAH-Tg58RuPp (red) mice.


SPLENIC CELLULARITY
C57BL/6J
MHOAH
MHOAH-Tg58RuPp
CELLS/SPLEEN (1 X 10^6)
600
500
400
300
200
100
0
NON-IMMUNIZED
IMMUNIZED

This increase in splenic cellularity of sickle cell mice compared to control mice cellularity may explain in part the increase in PFCs found in sickle cell mice. The percent increase in splenic cellularity between the immunized MHOAH-Tg58RuPp mice is less than the percent increase in the non-immunized mice. These data establish that these transgenic sickle cell mice are not immunodeficient when responding to SRBCs, but are in fact capable of responding with greater numbers of immunoglobulin secreting cells during both primary and secondary responses, with the exception of the primary IgG PFC.

Leukocyte Subset Analysis

Non-immunized sickle cell mice have approximately a threefold increase in splenic cellularity (Table 5 and Figure 2) over both MHOAH and C57BL/6J mice ($p<0.001$). Analysis of splenic leukocyte subsets (as defined by antibodies to cell surface molecules, Table 6) shows that sickle cell mice have a numerical increase in all subsets compared with C57BL/6J and MHOAH mice (Figures 3 and 4). These data also show that the increased number of cells of each subset is generally proportional to the increase in splenic cellularity with the exception of the PMN and MAC subsets which are increased to a greater degree (~8- and ~6-fold, respectively). Spleens of sickle cell mice, MHOAH and C57BL/6J mice contain 91 x 10^6 B lymphocytes, 37 x 10^6 B lymphocytes and 35 x 10^6 B lymphocytes, respectively, ($0.01<p<0.02$). When compared to the C57BL/6J subset values, the sickle cell B lymphocyte subset is expanded (~3-fold) proportionally to the increase in splenic cellularity.

Table 5. Results of Splenic Leukocyte Subset Analysis (x 10^6).

	C57BL/6J	MHOAH	MHOAH-Tg58	p value[¶]
Spleen (mg)	73 ± 4.3^{i}	85 ± 2.4^{h}	264 ± 22.7^{g}	$p < 0.001^{\S}$
No. Cells/Spleen	110 ± 5.4^{i}	122 ± 4.4^{h}	374 ± 41.0^{g}	$p < 0.001^{\S}$
Total B Cells	35 ± 5.3^{e}	37 ± 4.2^{e}	91 ± 18.5^{e}	$0.01 < p < 0.02^{\S}$
Total T Cells	15 ± 2.7^{e}	12 ± 1.5^{e}	33 ± 6.4^{e}	$0.01 < p < 0.02^{\S}$
Total $CD4^+$ Cells	11 ± 1.6^{f}	8 ± 1.4^{e}	26 ± 6.1^{e}	$0.005 < p < 0.01$
Total $CD8^+$ Cells	7 ± 1.0^{f}	3 ± 0.30^{e}	8 ± 1.6^{e}	$0.002 < p < 0.005$
CD4/CD8 Ratio	1.6 ± 0.13^{f}	2.5 ± 0.30^{e}	3.3 ± 0.62^{e}	not significant
Total NKs	1 ± 0.22^{a}	3 ± 1.5^{b}	8 ± 2.8^{a}	$0.1 < p < 0.2$
Total PMNs	3 ± 0.69^{e}	2 ± 0.42^{b}	15 ± 3.9^{e}	$0.01 < p < 0.02$
Total MACs	4 ± 0.80^{e}	5 ± 0.95^{d}	29 ± 5.7^{e}	$p < 0.001^{\S}$

Data are presented as the mean ± S.E.M. a, n=7; b, n=8; c, n=9; d, n=10; e, n=11; f, n=12; g, n=14; h, n=16; i, n=17.
[¶] All p values are for MHOAH vs.Tg58 unless otherwise noted. § denotes p value is the same for MHOAH vs. Tg58 and C57BL/6J vs. Tg58; C57BL/6J vs. Tg58: CD4+ $0.02 < p < 0.05$; CD8+ not significant; CD4/CD8 Ratio $0.01 < p < 0.02$; PMNs $0.002 < p < 0.005$ and NKs $0.02 < p < 0.05$.

Table 6. Antibodies, CD Designations and Cell Surface Molecules.

ANTIBODY	MOLECULE	CELL TYPE	REFERENCE
CD45R	CD45 Antigen	early pro- to mature B cell	Rolink and Melchers 1991
CD90 (Thy 1)	Thy 1 Antigen	mature thymocytes peripheral T cells	Ledbetter *et al.* 1980
CD4	CD4 Antigen	thymocyte subset T helper cells	Scollay *et al.* 1988
CD8	CD8 Antigen	thymocyte subset T cytotoxic cells	Scollay *et al.* 1988
F4/80	F4/80 Antigen	mature macrophages activated monocytes	Lee *et al.* 1985
NK 1.1	NK Antigen	natural killer cells	Glimicher *et al.* 1977
MDA	Myeloid Differentiation Antigen	polymorphonuclear neutrophils	Hestdal *et al.* 1991

Figure 3. Lymphocyte Subsets.

Mean cell number (x 10^6) per lymphocyte subset in C57BL/6J (yellow), MHOAH (blue), and MHOAH-Tg58RuPp (red) mice.

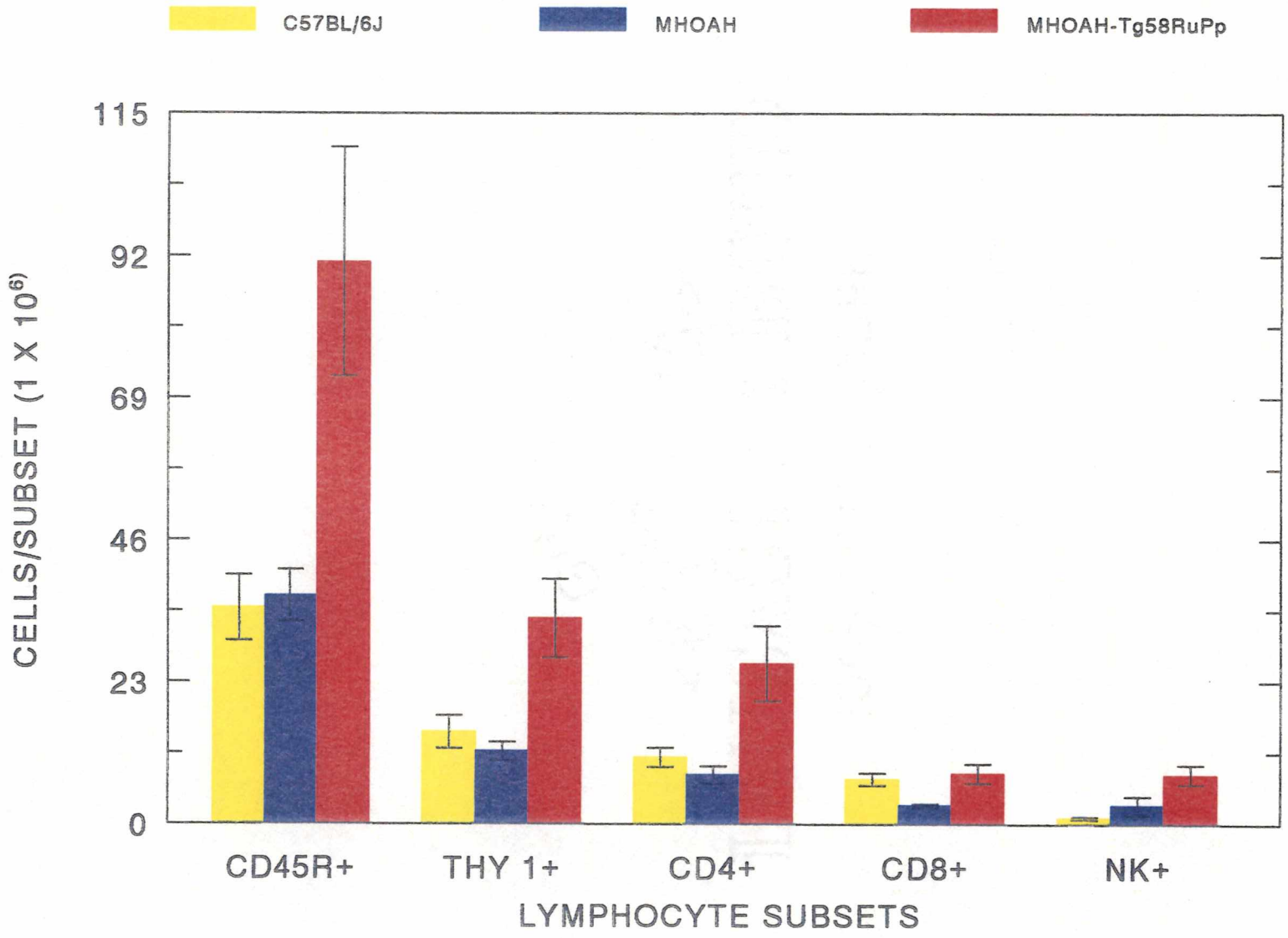
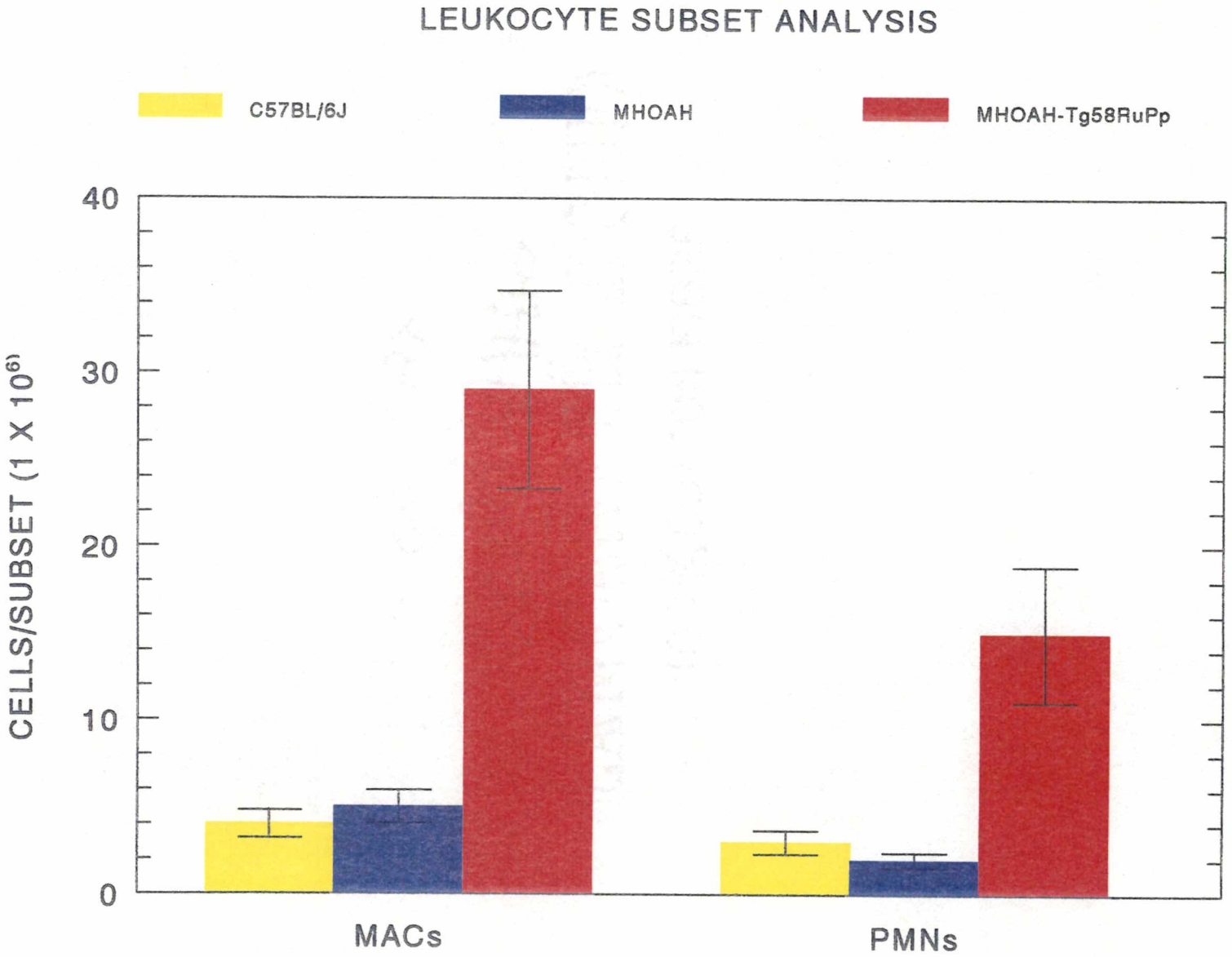

C57BL/6J
MHOAH
MHOAH-Tg58RuPp
CELLS/SUBSET (1 X 10⁶)
115
92
69
46
23
0
CD45R+
THY 1+
CD4+
CD8+
NK+
LYMPHOCYTE SUBSETS

Figure 4. MAC and PMN Subsets.

Mean cell number ($x10^6$) of the MAC and PMN subsets in C57BL/6J (yellow), MHOAH (blue), and MHOAH-Tg58RuPp (red) mice.


LEUKOCYTE SUBSET ANALYSIS
C57BL/6J
MHOAH
MHOAH-Tg58RuPp
CELLS/SUBSET (1 X 10⁶)
40
30
20
10
0
MACs
PMNs

The T cell subset (~2-fold) [including both CD4+ (~2-fold) and CD8+ (1.1-fold) T cells], is expanded in sickle cell mice but not to the same extent as the expansion in splenic cellularity. The ratio of splenic CD4+ to CD8+ cells in normal mice is 2 to 1 (Scollay *et al.* 1988). Sickle cell mice have a CD4+/CD8+ ratio of 3.3 to 1. This ratio, although higher it is not statistically significantly different than that found in MHOAH mice (2.5 to 1). The CD4+/CD8+ ratio is higher in sickle cell mice than in the C57BL/6J mice (1.6 to 1), and these values are statistically significantly different ($0.01<p<0.02$).

Sickle cell NK (~8-fold), PMN (~5-fold) and MAC (~7-fold) subsets are expanded to a greater degree than can be accounted for by the expansion in splenic cellularity (~3-fold).

Splenic and Peripheral Blood Subset Comparison

Peripheral blood subsets of sickle cell mice show the same general increase in absolute numbers shown by the splenic subsets, but there was more variability in the peripheral blood subsets than in the splenic subsets (Table 7). Several sickle cell subsets [B cell (4.6×10^6), T cell (2.3×10^6) and CD4 (10×10^3)] have more cells than either MHOAH [B cell (2.5×10^6), T cell (1.2×10^6), and CD4 (4×10^3)] or C57BL/6J [B cell (3.3×10^6), T cell (1.1×10^6), CD4 (6.1×10^3)]. The CD8+ subset in peripheral blood of sickle cell mice (3.2×10^3) has fewer cells than the CD8+ subset of C57BL/6J mice (4.7×10^3) and the CD8+ subset of MHOAH mice has the least number of cells (1.7×10^3).

Table 7. Comparison of Leukocyte Subsets in Spleen and Peripheral Blood.

		Strain		
		C57BL/6J	MHOAH	MHOAH-Tg58
B CELLS (10^6)	SPL	35^g	37^g	91^g
	PBL	3.3^d	2.5^d	4.6^c
T CELLS (10^6)	SPL	15^g	12^g	33^g
	PBL	1.1^d	1.2^d	2.3^c
CD4	SPL	11×10^{6h}	8×10^{6g}	26×10^{6g}
	PBL	6.1×10^{3d}	4×10^{3d}	10×10^{3c}
CD8	SPL	7×10^{6h}	3×10^{6g}	8×10^{6g}
	PBL	4.7×10^{3d}	1.7×10^{3d}	3.2×10^{3c}
NK	SPL	1×10^{6c}	3×10^{6d}	8×10^{6c}
	PBL	950^a	400^a	900^a
PMN (10^6)	SPL	3^g	2^d	15^e
	PBL	1.9^d	3.4^d	3.1^c
MACS (10^5)	SPL	40^g	50^f	290^g
	PBL	0.1^d	1.3^c	0.9^b

Data are presented as means. a, n = 5; b, n = 6; c, n = 7; d, n = 8; e, n = 9; f, n = 10; g, n = 11 and h, n = 12. SPL-spleen and PBL-peripheral blood.

The NK subset in peripheral blood (900 cells/mouse) of sickle cell mice and C57BL/6J, (950 cells/mouse) mice, was larger than in MHOAH (400 cells/mouse) mice. Two peripheral blood subsets, PMN and MACs, in sickle cell mice are approximately equal to that in MHOAH but are larger than that in C57BL/6J mice. Sickle cell and MHOAH PMN subsets have 3.1×10^6 and 3.4×10^6 cells/mouse, respectively, and C57BL/6J has 1.9×10^6 cells/mouse. MAC subsets in peripheral blood of sickle cell mice have 0.9×10^5 cells/mouse, MHOAH mice have 1.3×10^5 cells/mouse, and C57BL/6J mice have 0.1×10^5 cells/mouse.

CHAPTER 4

Discussion

MHOAH-Tg58RuPp mice immunized with SRBCs have a two-fold increase in splenic cellularity compared with immunized MHOAH and C57BL/6J mice. The hemolytic plaque assay of Jerne and Nordin (1963) was used as modified by Mishell and Dutton (1966) to detect the number of plaque-forming cells (PFCs) responding to sheep red blood cells in a primary and secondary immune response. A primary response is the immune system's response the first time an antigen is encountered. The primary antibody response is characterized predominantly by immunoglobulin M (IgM). The secondary response occurs when an antigen is encountered a second time. The secondary antibody response is characterized by immunoglobulin G (IgG). The secondary antibody response produces much more IgG and produces it in a shorter period of time following exposure (Roitt 1989). MHOAH-Tg58RuPp mice have an elevated number of primary and secondary IgM PFCs relative to both C57BL/6J and MHOAH mice. The increase in number of primary IgM PFCs corresponds to the increase in splenic cellularity (Table 3 and 4), and the increase in splenic cellularity is a result of the increased splenic hematopoiesis (Popp *et al.* 1993). The increase in number of secondary IgM PFCs in MHOAH-Tg58RuPp mice, being greater than the increase in splenic cellularity, cannot be explained by the increase in splenic cellularity.

Primary IgG PFCs seem to be lower in MHOAH-Tg58RuPp mice than in C57BL/6J and MHOAH mice. Secondary IgG PFCs seem to be elevated in MHOAH-Tg58RuPp mice relative to control mice. This data may indicate that a population of cells

that secrete IgG during a primary response to antigen that are present in C57BL/6J and MHAOH mice is absent from MHOAH-Tg58RuPp mice. More experiments will need to be conducted to determine if the differences observed in IgG PFCs are statistically significant. Even though MHOAH-Tg58RuPp mice have a lower number of primary IgG PFCs, the total number of PFCs in MHOAH-Tg58RuPp mice is much higher than in either of the control mice. These studies show that MHOAH-Tg58RuPp, transgenic sickle cell mice, are not immunodeficient in their ability to respond to SRBCs.

In order to correlate results of the immune response with possible perturbations in immunocompenent or immune-related cells, leukocyte subsets of the spleen and peripheral blood were examined by flow cytometric analysis. Leukocyte subset analyses define the leukocyte perturbations that exist in the spleen and peripheral blood of these transgenic sickle cell mice. Leukocyte subset analyses, which included B and T lymphocytes, CD4+, CD8+, PMN, MAC and NK subsets, show that all of these are expanded in the spleen of sickle cell mice. These subsets are also expanded in peripheral blood, with the exception of the CD8+ and NK subsets. Expansion of the B lymphocyte subset, which was indicated by the increased PFCs, was confirmed by analysis with the antibody CD45R. Expansion of the following sickle cell subsets relative to MHOAH controls occurred: B cell, T cell, CD4+, CD8+ and NK, corresponds to the 3-fold expansion in splenic cellularity ($p<0.001$). Phagocytic subsets, PMNs and MACs, which are expanded to a greater degree may be partially explained by the need to remove the increased number of misshapen and damaged red cells. These two subsets share a common hematopoietic progenitor, colony forming unit granulocyte/macrophage, (Ikuta *et al.* 1992) which is

separate from the lymphocyte progenitor; this could explain the preferential expansion of PMN and MAC subsets.

T lymphocyte subsets in sickle cell mice are not expanded to the same extent as splenic cellularity. When a comparison between the T subset of MHOAH and C57BL/6J is made it can be seen that the T cell subsets are reduced. This reduction may reflect upon the presence of the high oxygen affinity hemoglobins in the MHOAH mice. The reduction in the T cell subsets may reflect the presence of the high affinity hemoglobins. When MHOAH-Tg58RuPp T cell subsets are compared to the C57BL/6J control the expansion of the T cell subsets in the MHOAH-Tg58RuPp mice is lower than would be expected if the increase in splenic cellularity was used to predict the amount of expansion expected . This reduction in T lymphocytes, but not B lymphocytes, may also reflect a division of hematopoietic progenitors between T and B lymphocytes (Ikuta *et al.* 1992) or the presence/absence of a growth factor which influences only one of these two subsets. Comparison of the NK subset of MHOAH relative to C57BL/6J reveals an elevation greater than the increase in cellularity. Therefore, expansion of the NK subset may also be influenced by the presence of the high oxygen affinity hemoglobins and influenced only slightly by sickle cell pathobiology (which would explain why this subset is not expanded significantly over the NK subset of MHOAH mice).

Leukocyte subsets seem to separate into three groups relative to size. The physiological conditions generated by the presence of high oxygen affinity hemoglobins affects T lymphocytes and NK cells. The presence of polymerized sickle Hb affects MACs

and PMNs. The increased hematopoiesis accounts for the elevated number of B lymphocytes.

This mouse model of sickle cell disease seems to be a promising research tool for investigators interested in many areas including development of T and B cells, and NK cells.

Future avenues of investigation could include phospholipid analysis of inner and outer membrane leaflets of erythrocytes from MHOAH-Tg58RuPp mice and analysis of complement factors in the classical and alternative pathways. In the outer leaflet of the cell membrane in normal human erythrocytes 12% of the phospholipids is phosphatidyl-ethanolamine. There is no phosphatidylserine in the outer leaflet of a normal erythrocyte membrane (Tomasko and Chudwin 1988). However, Lubin *et al.* (1981) have shown that the outer leaflet of ICSs has 22% phosphatidylethanolamine and 2% phosphatidylserine. An analysis of the phospholipid content of the outer leaflet of MHOAH-Tg58RuPp erythrocytes could be done to determine if the same type of phospholipid changes occur in the ISCs. A conformation of a change such as this in erythrocytes of MHOAH-Tg58RuPp mice would establish further that these mice are an excellent model of human sickle cell disease.

LIST OF REFERENCES

LIST OF REFERENCES

Ades, E.W., A. Hinson and S.K. Morgan. 1980. Immunological Studies in Sickle Cell Disease. *Clin Immunol Immunopath* 17: 459-462.

Ballester, O.F., J.M. Abdallah and A. S. Prasad. 1986. Lymphocyte Subpopulation Abnormalities in Sickle Cell Anemia: A Distinctive Pattern From That of AIDS. *Am J Hematol* 21: 23-27.

Barrett-Connor, E. 1971. Bacterial Infection and Sickle Cell Anemia. An Analysis of 250 Infections in 166 Patients and a Review of the Literature. *Medicine* 50/2: 97-112.

Bjornson, A.B., J.S. Lobel, P.I. Magnafichi and B.C. Lampkin. 1981. Restoration by Normal Human Immunoglobulin G of Deficient Serum Opsonization for *Streptococcus pneumoniae* in Sickle Cell Disease. *Infection and Immunity* 33/2: 636-640.

Bjornson, A.B., J.S. Lobel and K.S. Harr. 1985. Relation Between Serum Opsonic Activity for *Streptococcus pneumoniae* and Complement Function in Sickle Cell Disease. *J Infect Diseases* 152/4: 701-709.

Bjornson, A.B. and J.S. Lobel. 1986. Lack of a Requirement for the Fc Region of IgG in Restoring Pneumococcal Opsonization via the Alternative Complement Pathway in Sickle Cell Disease. *J Infect Diseases* 154/5: 760-769.

Bjornson, A.B. and J.S. Lobel. 1987. Direct Evidence that Decreased Serum Opsonization of *Streptococcus pneumoniae* Via the Alternative Pathway in Sickle Cell Disease is Related to Antibody Deficiency. *J Clin Invest* 79: 388-398.

Buchanan, G.R., S.J. Smith, C.A. Holtkamp and J. P. Fuseler. 1983. Bacterial Infection and Splenic Reticuloendothelial Function in Children with Hemoglobin SC Disease. *Pediatrics* 72/1: 93-98.

D'Surney, S.J. and R.A. Popp. 1992. Oxygen Association-Dissociation and Stability Analysis on Mouse Hemoglobins With Mutant α- and β-Globins. *Genetics* 132: 545-551.

Fabry, M.E. 1993. Transgenic Animal Models of Sickle Cell Disease. *Experientia* 49: 28-36.

Glimcher, L., F.W. Shen and H. Cantor. 1977. Identification of a Cell-Surface Antigen Selectively Expressed on the Natural Killer Cell. *J Exp Med* 145: 1-9.

Henry, C. 1980. Hemolytic Plaque Assays. In B.B. Mishell and S.M. Shiigi (Eds.), *Selected Methods in Cellular Immunology*. W.H. Freeman and Co. San Francisco. pp. 69-123, 261-264, 443-456.

Ikuta, K. , N. Uchida, J. Friedman, and I.L. Weissman. 1992. Lymphocyte Development From Stem Cells. *Annu Rev Immunol* 10: 759-783.

Ingram, V.M. 1957. Gene Mutations in Human Haemoglobin: The Chemical Difference Between Normal and Sickle Cell Haemoglobin. *Nature* 180: 326-328.

Jerne, N.K. and A.A. Nordin. 1963. Plaque Formation in Agar by Single Antibody-producing Cells. *Science* 140: 405.

Johnson, R.M., J.K. Dzandu and J.A. Warth. 1986. The Phosphoproteins of the Sickle Erythrocyte Membrane. *Arch Biochem Biophys* 244/1: 202-210.

Kaplan, J., S. Sarnaik, J. Gitlin and J. Lusher. 1984. Diminished Helper/supperssor Lymphocyte Ratios and Natural Killer Activity in Recipients of Repeated Blood Transfusions. *Blood* 64/1: 308-310.

Lewis, S.E., F.M. Johnson, L.C. Skow, D.M. Popp, L.B. Barnett and R.A. Popp. 1985. A Mutation in the β-Globin Gene Detected in the Progeny of a Female Mouse Treated with Ethylnitrosourea. *Proc Natl Acad Sci* USA 82: 5829-5831.

Lubin, B. , D. Chiu, J. Bastacky, B. Roelofsen and L.L.M. van Deenen. 1981. Abnormalities in Membrane Phospholipid Organization in Sickled Erythrocytes. *J Clin Invest* 67: 1643-1649.

Mishell, R.I. and R.W. Dutton. 1966. Immunization of Normal Mouse Spleen Cell Suspensions *in vitro*. *Sci* 153: 1004.

Nagel, R.L. 1994. Lessons from Transgenic Mouse Lines Expressing Sickle Hemoglobin. *PSEBM* 205: 274-281.

Perutz, M. F., A.M. Liquori and F. Eirich. 1951. X-ray and Solubility Studies of Haemoglobin of Sickle-Cell Anaemia Patients. *Nature* 167: 929-931.

Perutz, M. F. and J.M. Mitchison. 1950. State of Haemoglobinin Sickle-Cell Anaemia. *Nature* 166: 677-679.

Popp, R.A., S.G. Shinpock, K.D. Russell, T.L. Poole and E.M. Rubin. 1993. *In vivo* Sickling of Red Cells and Pathobiology in a Newly Developed Transgenic Hb $S^{Antilles}$ Line of Mice. National Sickle Cell Disease Program 18:3A.

Popp, R.A., S.L. Bloch, S.G. Shinpock and D.M.Popp. 1990a. Expression of the Globin Genes and Hematopoiesis in β-thalassemic Mice. In: Molecular Biology of Erythropoiesis. J.L. Ascensao, E.D. Zanjani, M. Tavassoli, A.S. Levine and F.R. MacKintosh. (Eds). pp161-176. Plenum Press, New York, NY.

Popp, R.A., S.G. Shinpock and D.M. Popp. 1990b. Immunodeficiency of *β*-Thalassemic Mice. *Annals NY Acad Sci* 612: 539-539.

Popp, R.A., E.G. Bailiff, L.C. Skow, F.M. Johnson and S.E. Lewis. 1983. Analysis of a Mouse α-Globin Gene Mutation Induced by Ethylnitrosourea. *Genetics* 105: 157-167.

Powars, D.R. and W.Y. Wong. 1993. Sickle Cell Anemia (SS): Phenotypic Lymphocyte Subsets. 18th Annual Sickle Cell Meeting. Philadelphia, PA. Abstract.

Robinson, M.G. and R.J. Watson. 1966. Pneumococcal Meningitis in Sickle-Cell Anemia. *N Eng J Med* 274: 1006-1008.

Roitt, I.M. 1989. *Essential Immunology*. 6th Edition. Blackwell Scientific Publications. Oxford, United Kingdom.

Rubin, E.M., H.E. Witkowska, E. Spangler, P. Curtin, B.H. Lubin, N. Mohandas and S.M. Clift. 1991. Hypoxia-induced *In Vivo* Sickling of Transgenic Mouse Red Cells. *J Clin Invest* 87/2: 639-647.

Scollay, R., A. Wilson, A. D'Amico, K. Kelly, M. Egerton, M. Pearse, L. Wu and K. Shortman. 1988. Developmental Status and Reconstitution Potential of Subpopulations of Murine Thymocytes. *Immunological Rev* 104: 81-120.

Sergeant, G.R. 1992. *Sickle Cell Disease*. 2nd Edition. Oxford University Press. Oxford, United Kingdom.

Titus, J.A., F.D. Finkelman, D.A. Stephany, J.F. Jones and D.M. Segal. 1984. Quantitative Analysis of Fc_γ Receptors on Murine Spleen Cell Populations by Using Dual Parameter Flow Cytometry. *J Immunol* 133/2: 556-561.

Tomasko, M. and D. Chudwin. 1988. Complement Activation in Sickle Cell Disease: A Liposome Model. *J Lab Clin Med* 112: 248-253.

Venkataraman, M. and M.P. Westerman. 1985. B-Cell Changes Occur in Patients with Sickle Cell Anemia. *Am J Clin Pathol* 84: 153-158.

VITA

Melissa Elizabeth Overcash was born October 3, 1965 in Charlotte, North Carolina. She attended East Gaston High School, Mt. Holly, North Carolina and graduated in 1983. She then attended Belmont Abbey College, Belmont, North Carolina where she received a Bachelor of Science degree in May of 1989. She then moved to Oak Ridge, Tennessee to attend graduate school at the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences located in the Biology Division of Oak Ridge National Laboratory, Oak Ridge, Tennessee. She received her Masters of Science degree with a concentration in Immunology in May of 1995 for research performed under the direction of Dr. Raymond Arthur Popp.