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**AUTOIMMUNITY, IMMUNE DEFICIENCY AND CANCER: MULTIPLE
ROLES OF THE PROTEIN TYROSINE PHOSPHATASE SHP-1.**

By

Melissa J. Joliat

B.S. College of the Atlantic, 1992

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Biochemistry and Molecular Biology)

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The University of Maine

December, 2001

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**AUTOIMMUNITY, IMMUNE DEFICIENCY AND CANCER: MULTIPLE
ROLES OF THE PROTEIN TYROSINE PHOSPHATASE SHP-1.**

By Melissa J. Joliat

Thesis Advisor: Dr. Leonard D. Shultz

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
(in Biochemistry and Molecular Biology)
December, 2001

One of a large number of mutant mice used in immunological research, the “motheaten” mouse was the first model of a specific protein tyrosine phosphatase deficiency. Mice carrying one of two allelic mutations at the “motheaten” locus have severe systemic autoimmunity and immune dysfunction as a result of mutations in the hematopoietic-cell phosphatase (*Hcph*) gene, which encodes the protein tyrosine phosphatase SHP-1. Studies using “motheaten” (*me/me*) and “viable motheaten” (*me^v/me^v*) mice have increased the understanding of numerous signaling pathways in immune and hematopoietic cells. A number of studies on SHP-1 function in normal and pathologic states are described here.

Homozygous *me^v/me^v* mice have an increased percentage of autoantibody associated B-1 B-cells that express the cell surface glycoprotein CD5. To investigate the hypothesis that absence of CD5 in *me^v/me^v* mice will result in decreased systemic autoimmunity, we created a stock of *CD5^{null} me^v/me^v* mice. These mice have a longer lifespan than *me^v/me^v* mice, associated with reduced pulmonary inflammatory disease,

splenic macrophage numbers, and serum IgM levels. However, autoantibodies against dsDNA and histone proteins were not significantly reduced. These studies suggest that CD5 expression is not required for autoantibody production, but otherwise indicate a role for CD5 in the development of immunopathologic lesions in *me^y/me^y* mice.

Dysregulated macrophage populations in *me^y/me^y* mice presumably have secondary effects on other cell types. To examine these effects, as well as the primary results of SHP-1 deficiency in macrophages, we developed a stock of mice transgenic for a dominant-negative form of SHP-1, under control of a macrophage specific promoter. The catalytically inactive dominant-negative protein should occupy SH2 binding sites, blocking the recruitment of functional wild-type SHP-1.

SHP-1 plays a putative role in oncogenesis. To substantiate this role, we have monitored tumor development in aged *+/me* and *+/me^y* mice. Preliminary studies do not support the hypothesis that a spontaneous chondroblastic osteosarcoma that occurred in an aging *+/me^y* mouse was caused by loss of SHP-1 expression. Nonetheless, we describe the transplantable cell-line derived from this tumor, which mimics the process of endochondral ossification *in vivo* and is a potentially valuable model for studies of osteosarcoma and bone biology.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES	ix
LIST OF FIGURES.....	x

Chapter

1. THE MOLECULAR BASES OF SPONTANEOUS IMMUNOLOGICAL MUTATIONS IN THE MOUSE AND THEIR HOMOLOGOUS HUMAN DISEASES.....	1
Introduction	1
Spontaneous Immunological Mutations in the Mouse	7
Severe combined immune deficiency (<i>Prkdc^{scid}</i>)	7
Nude (<i>Foxn1^{nu}</i>).....	8
Scurfy (<i>Foxp3^{sf}</i>)	10
Lymphoproliferation (<i>Tnfrsf6^{lpr}</i>) and generalized lymphoproliferative disease (<i>Tnfrsf6^{gld}</i>)	12
Dominant white spotting (<i>Kit^W</i>) and steel (<i>Kitt^{Sl}</i>)	14
X-linked immune deficiency (<i>Btk^{xid}</i>).....	15
Alymphoplasia (<i>Map3k14^{aly}</i>)	17
Beige (<i>Lyst^{bs}</i>)	19
Osteopetrosis (<i>Csf1^{op}</i>).....	21
Defective lipopolysaccharide response (<i>Tlr4^{lps-d}</i>)	22

Other mutations {microphthalmia (<i>Mitf^{mi}</i>), wasted (<i>Eef1a2^{wst}</i>), hairless (<i>hr</i>)}	24
Motheaten (<i>Hcph^{me}</i>).....	26
Conclusion	28
 2. SHP-1 PROTEIN TYROSINE PHOSPHATASE AND THE “MOTHEATEN” MOUSE	30
 3. ABSENCE OF CD5 DRAMATICALLY REDUCES PROGRESSION OF PULMONARY INFLAMMATORY LESIONS IN SHP-1 PROTEIN-TYROSINE PHOSPHATASE-DEFICIENT “VIABLE MOTHEATEN” MICE	36
Abstract	36
Introduction	37
Materials and Methods.....	40
Mice.....	40
Histopathology	41
Flow cytometric analysis.....	42
Ig levels	43
Autoantibody determinations by ELISA.....	43
Hematology	44
Spleen cell lysates and immunoblotting.....	44
Bone marrow macrophage cultures.....	45
Statistics.....	46

Results.....	46
Longevity.....	46
Immunopathology	48
Flow cytometry analyses of cell populations	55
Peripheral blood analysis.....	58
Serum Ig levels	61
Autoantibody assays.....	63
Anti-phosphotyrosine western blotting	64
Bone marrow macrophage cultures.....	65
Discussion	68

4. GENERATION OF C57BL/6J MICE CARRYING A TRANSGENE FOR MACROPHAGE SPECIFIC EXPRESSION OF DOMINANT NEGATIVE SHP-1 (C453S).....	74
Introduction	74
Materials and Methods.....	77
Construction of SHP-1 dominant negative transgene for expression in macrophages.	77
Transgenic mice	77
Hematology	78
Culture of bone marrow macrophages	78
Immunoblotting	79
Results and Discussion	79

5. ESTABLISHMENT AND CHARACTERIZATION OF A NEW CELL LINE

(MOS-J) FROM A SPONTANEOUS C57BL/6J MOUSE OSTEOSARCOMA. 87

Abstract 87

Introduction 87

Materials and Methods..... 89

Source of cancer tissue 89

Histopathology 89

Establishment of the MOS-J cell line..... 90

Growth rate 90

Cell culture characteristics 91

Alkaline phosphatase detection *in situ* 91

Alizarin red staining..... 92

Support of osteoclastogenesis..... 92

Oncogenicity in mice 93

Transmission electron microscopy 93

Amputation of affected limb..... 94

Results..... 94

Histopathology of the original tumor..... 94

Cell culture characteristics 95

Alkaline phosphatase activity 97

Alizarin red staining..... 97

Osteoclast growth assay 97

Oncogenicity <i>in vivo</i>	100
Ultrastructural findings of transplanted tumors	100
Discussion	108
CONCLUSION	112
REFERENCES	113
BIOGRAPHY OF THE AUTHOR	166

LIST OF TABLES

Table 1.1. The molecular bases of spontaneous immunological mutations in the mouse.	3
Table 1.2. Human homologous diseases and uses of mouse models in the study of human disease and immunological function.	5
Table 3.1. Flow cytometric analyses of spleen cells from 9 – 12 wk-old B6;129-CD5 ^{-/-} <i>me^v/me^v</i> mice.	56
Table 3.2. Peripheral blood analysis of 9 – 12 wk-old CD5 ^{-/-} <i>me^v/me^v</i> mice.	61
Table 3.3. Flow cytometry analysis of bone marrow cultures.....	66

LIST OF FIGURES

Figure 2.1. Structure of SHP-1	31
Figure 2.2. Functions of SHP-1 in regulating signals through the B-cell receptor.....	32
Figure 2.3. Functions of SHP-1 in regulating signals through the T-cell receptor.....	33
Figure 3.1. Structure of CD5.	39
Figure 3.2. Cumulative percent survival.	47
Figure 3.3. Photomicrographs of lung of CD5 ^{-/-} <i>me^y/me^y</i> mice.....	51
Figure 3.4. Photomicrographs of spleen of CD5 ^{-/-} <i>me^y/me^y</i> mice.....	52
Figure 3.5. Photomicrographs of liver of CD5 ^{-/-} <i>me^y/me^y</i> mice.....	53
Figure 3.6. Photomicrographs of kidney and stomach of CD5 ^{-/-} <i>me^y/me^y</i> mice.....	54
Figure 3.7. Flow cytometry analysis of B-1 B cells.....	57
Figure 3.8. New methylene blue (NMB) staining for reticulocytes.....	59
Figure 3.9. Serum immunoglobulin levels	62
Figure 3.10. Autoantibody determination by ELISA.....	64
Figure 3.11. Characterization of tyrosine phosphorylation	65
Figure 3.12. Expression of CD5 on bone marrow cells	67
Figure 4.1. The dominant negative SHP-1 mutation C453S	76
Figure 4.2. Construction of SHP-1 C453S transgene	80
Figure 4.3. PCR typing of SHP-1 C453S transgenic founders.....	83
Figure 4.4. Peripheral blood analysis of SHP-1 C453S transgenic mice.....	84
Figure 4.5. Anti-SHP-1 western blot.....	85
Figure 4.6. Anti-ptyr western blot.....	86

Figure 5.1. Growth rate of MOS-J cells in culture.....	95
Figure 5.2. Morphological features of MOS-J cells.....	96
Figure 5.3. Cytochemical features of MOS-J cells	98
Figure 5.4. Pelvis/femur radiographs of mice with transplanted tumors	102
Figure 5.5. Histological appearance of transplanted tumors	104
Figure 5.6. Transmission EM of transplanted tumor	106

Chapter 1

THE MOLECULAR BASES OF SPONTANEOUS IMMUNOLOGICAL MUTATIONS IN THE MOUSE AND THEIR HOMOLOGOUS HUMAN DISEASES ¹

Introduction

Spontaneous genetic mutations that disrupt development or regulation of the immune system in inbred mouse strains provide unique models for the study of immune deficiency and autoimmune diseases of humans. While these mouse mutations have been widely utilized to increase our understanding of the immune system in normal and pathological states, recent determination of the molecular bases of many of these mutations has provided even deeper insight into the underlying biology and biochemistry of the mammalian immune system. A number of the mouse genes disrupted by spontaneous mutation have human homologues, and mutations in many of the human genes result in immunological diseases. In these cases, immunological mutations in mice provide an especially valuable resource to study mechanisms of disease and to investigate experimental interventions that may lead to treatments for the human disease.

In recent years, advances in transgenic and gene targeting technology have allowed generation of mice with genetic alterations specifically targeted to selected genes or regions of genes, allowing focused analysis of their products and functions. While these technologies have become powerful tools for scientific research, they commonly rely on identification and cloning of a gene prior to creating a targeted mutation of that

gene. While targeted mutation of a specific gene often results in an imperceptible or unexpected phenotype, spontaneous mutants are selected by discernable phenotypes of interest. Investigation into the genetic basis of many of these spontaneous mutations has led to the identification of previously unknown, novel genes.

The intent of this review is not to provide exhaustive detail about the phenotypes of mutant mice. The phenotypes of many of these mutants have been reviewed previously (1), and references to available reviews specific to each mutant will be provided. This review has been written as an overview of spontaneous mouse mutations resulting in immunodeficient or autoimmune phenotypes that have been defined at the molecular level (summarized in Table 1.1). Human diseases with homology to these mouse mutations are discussed, and the uses of these mice in research on human disease and immunobiological function are also discussed (summarized in Table 1.2).

Genetic nomenclature can be complex. Gene symbols are changed as loci are identified at the molecular level and gene families are determined. The current approved symbol for each mouse gene is provided in the heading for the individual mutations, as well as in Table 1.1. The symbol for the homologous human gene is provided in Table 1.2, and is included in the text. General background of nomenclature changes is provided in the text, and the most commonly known name for each gene and mutation is used in subsequent discussion.

¹ Based on: Joliat MJ, Shultz LD. 2001. The molecular bases of spontaneous immunological mutations in the mouse and their homologous human diseases. (Review) *Clin. Immunol.* 101(3):113-129.

Table 1.1. The molecular bases of spontaneous immunological mutations in the mouse.

Allele Name (Original Symbol)	Gene Symbol	Gene Name	Mouse Chr	Phenotype
severe combined immunodeficiency (<i>scid</i>)	<i>Prkdc</i>	DNA activated protein kinase, catalytic subunit	16	Severe immune deficiency caused by absence of both B and T cells, radiosensitivity
nude (<i>nu</i>)	<i>Foxn1</i>	forkhead box n1 (forkhead/winged helix family of transcription factors)	11	Hairless, athymic, T-cell deficiency
scurfy (<i>sf</i>)	<i>Foxp3</i>	forkhead box P3 (forkhead/winged helix family of transcription factors)	X	Thrombocytopenia, increased numbers of CD4+/CD8- T lymphocytes, extensive multi-organ infiltration, and elevation of numerous cytokines
motheaten (<i>me</i>),	<i>Hcph</i>	hematopoietic cell phosphatase	6	Severe immune deficiency, autoimmunity, x-ray resistance
lymphoproliferation (<i>lpr</i>)	<i>Tnfrsf6</i>	tumor necrosis family receptor superfamily, member 6	19	Lymphadenopathy, autoimmunity
generalized lymphoproliferative disease (<i>gld</i>)	<i>Tnfsf6</i>	tumor necrosis factor superfamily, member 6	1	Lymphadenopathy, autoimmunity
dominant spotting (<i>W</i>)	<i>Kit</i>	kit oncogene (stem cell factor receptor)	5	Defects in pigment forming cells, RBC's, mast cells and progenitor cells, impaired resistance to parasitic infection
steel (<i>sl</i>)	<i>Kitl</i>	kit ligand (stem cell factor)	10	Severe macrocytic anemia, mast cell deficiency
X-linked immunodeficiency (<i>xid</i>)	<i>Btk</i>	Bruton's agammaglobulinemia tyrosine kinase	X	Defective immune response to type II thymus-independent (TI-II) antigens, impaired immune response to some thymus-dependent (TD) antigens, and impaired lymphocyte response to B-cell mitogens
alymphoplasia (<i>aly</i>)	<i>Map3k14</i>	mitogen-activated protein kinase kinase kinase 14	11	Absence of lymph nodes and Peyer's patches

Allele Name (Original Symbol)	Gene Symbol	Gene Name	Mouse Chr	Phenotype
beige (<i>bg</i>)	<i>Lyst</i>	lysosomal trafficking regulator	13	Abnormal giant lysosomal granules in granule containing cells, defective granulocyte activity, NK cell deficiency, platelet storage pool deficiency
osteopetrosis (<i>op</i>)	<i>Csf1</i>	colony stimulating factor 1 (macrophage)	3	Osteoclast defects, macrophage deficiency, monocytopenia, defective bone remodeling
defective lipopolysaccharide response (<i>Lps^d</i>)	<i>Tlr4</i>	toll-like receptor 4	4	Defective response to bacterial endotoxin. Increased susceptibility to Gram-negative infection.
microphthalmia (<i>mi</i>)	<i>Mitf</i>	microphthalmia associated transcription factor	6	Developmental defects in melanocytes, osteoclasts and mast cells
wasted (<i>wst</i>)	<i>Eef1a2</i>	eukaryotic translation elongation factor 1 alpha 2	2	Tremor, progressive paralysis, lymphoid hypoplasia, death by 30 days of age
hairless (<i>hr</i>)	<i>hr</i>	hairless	14	High incidence of early onset leukemia, low cellular immune response, deficiency of splenic T _H cells.

Table 1.2. Human homologous diseases and uses of mouse models in the study of human disease and immunological function.

Mouse Mutation	Human Gene	Human Disease Homologues and Uses in Research on Human Diseases
severe combined immunodeficiency	<i>PRKDC</i> (Chr 8q)	Mutations in the human <i>PRKDC</i> gene have not been found associated with human SCID or any other disease. However, <i>scid</i> mice are widely used as hosts for normal and malignant human cells. They are also used as a model to study combined immune deficiency in humans and as a tool to decipher the normal biological role of DNA-PK.
nude	<i>WHN</i> (Chr 17q)	A mutation in this gene has been found in a family in Italy resulting in immune deficiency with absence of thymus and hair (OMIM 601705). These mice also serve as hosts for human solid tumors.
scurfy	<i>FOXP3</i> (Chr X)	Mutations in <i>FOXP3</i> are associated with the X-linked syndrome of immunodysregulation, polyendocrinopathy and enteropathy (IPEX) (OMIM 304930). May be used to study autoimmune disease development and molecular mechanisms involved in T cell regulation.
motheaten	<i>PTPN6</i> (Chr 12p)	No diseases are known to result from heritable mutations in <i>PTPN6</i> . Somatic mutations in this gene and defective gene expression have been associated with myeloid leukemia, T-cell lymphoma, polycythemia vera and breast cancers. Motheaten mice are also used to study the normal function of the SHP-1 phosphatase in the immune system.
lymphoproliferation	<i>TNFRSF6</i> (Chr 10q)	Mutations in <i>TNFRSF6</i> and <i>TNFSF6</i> are associated with autoimmune lymphoproliferative syndrome 1A (ALPS1A-also called Canale-Smith syndrome) and ALPS1B, respectively (OMIM 601859). Spontaneous somatic mutations in <i>TNFRSF6</i> have been found in cases of T-cell leukemia, multiple myeloma and non-Hodgkin's lymphoma. Study of these mice can also provide insights into the death receptor mediated system and its role in human disease.
generalized lymphoproliferative disease	<i>TNFSF6</i> (Chr 1q)	
dominant spotting	<i>KIT</i> (Chr 4q)	Germ line mutations in <i>KIT</i> have been found to cause human piebaldism (OMIM 172800). Somatic mutations, many of which cause constitutive activation of <i>KIT</i> , have been associated with mast cell neoplasia, cutaneous mastocytosis and tumors of the gastrointestinal stroma in humans.
steel	<i>KITLG</i> (Chr 12q)	
X-linked immunodeficiency	<i>BTK</i> (Chr X)	Bruton's X-linked agammaglobulinemia (XLA) (OMIM 300300) is caused by a variety of mutations in <i>BTK</i> . Mice with the <i>xid</i> mutation are also valuable tools for investigation of B cell-lineage development and signaling.

Mouse Mutation	Human Gene	Human Disease Homologues and Uses in Research on Human Diseases
alymphoplasia	<i>MAP3K14</i> (Chr 17q)	There are no known human diseases caused by mutations in <i>MAP3K14</i> . Mice homozygous for the <i>aly</i> mutation are valuable tools in the study of inflammatory processes involving NF- κ -B induction.
beige	<i>CHS1</i> (Chr 1q)	The <i>bg</i> mouse is the homologue of human Chediak-Higashi syndrome (OMIM 214500). Studies using the <i>bg</i> mouse may contribute to the understanding of the accelerated phase of Chediak-Higashi syndrome by helping to reveal the role of CTLA4 in T-cell regulation and lymphoproliferation.
osteopetrosis	<i>CSF1</i> (Chr 1p)	Osteopetrosis in humans has not been found associated with mutations in <i>CSF1</i> . However, <i>op</i> mice are a valuable resource in the study of osteopetrotic diseases and have contributed significantly to the understanding of normal bone biology
defective lipopolysaccharide response	<i>TLR4</i> (Chr 9q)	Mutations in <i>TLR4</i> have been associated with reduced response to inhaled LPS in humans. Study of mice carrying the <i>Lps^d</i> allele may lead to new approaches in the treatment of septic shock, endotoxin induced airway inflammation and other endotoxin mediated diseases.
microphthalmia	<i>MITF</i> (Chr 3p)	Mutations in <i>MITF</i> are associated with Waardenburg Syndrome type 2 (WS2) (OMIM 193510) and Tietz syndrome (OMIM 103500). Studies of <i>mi</i> mice can provide insights into mechanisms of melanocyte signaling and development.
wasted	<i>EEF1A2</i> (Chr 20q)	There are no human diseases known to be related to mutations in <i>EEF1A2</i> . Studies of <i>wst</i> mice have revealed mechanisms in the developmental regulation of protein translation.
hairless	<i>HR</i> (Chr 8p)	Mutations in the human <i>HR</i> gene are associated with congenital atrechia with papules (OMIM 209500).

Spontaneous Immunological Mutations in the Mouse

Severe combined immune deficiency (*Prkdc^{scid}*)

In 1983, Bosma *et al.* (2) reported a mutation (*scid*) in a colony of C.B-17 mice whose phenotype resembled human severe combined immune deficiency (SCID). Homozygosity for the *scid* mutation results in a deficiency of T and B lymphocytes with accompanying hypogammaglobulinemia and absence of both humoral and cell-mediated immune function (reviewed in (3)). Although C.B-17-*scid* mice have defects in adaptive immunity, many aspects of their innate immune function are unaltered ((4) and included references). C.B-17-*scid* mice have been widely used as hosts for human hematolymphoid cells, but intact innate immunity prevented long term survival of these cells. Backcrossing of the *scid* mutation onto the NOD/Lt strain background, which confers multiple defects in innate immunity, provided a *scid* mouse model in which survival of transplanted human cells was significantly increased (4). NOD/LtSz-*scid* mice are now a commonly used host for normal as well as malignant human hematolymphoid cells in many areas of research.

The *scid* mutation results in failure of V(D)J recombination (5-8) and also causes a defect in DNA double-strand break (DSB) repair (9, 10), which leads to increased radiosensitivity (11). These observations preceded the discovery that the mutation causing the *scid* phenotype was within the gene encoding the catalytic subunit of a DNA-dependent protein kinase (DNA-PK_{cs}) (12), now given the formal gene symbol *Prkdc*. DNA-PK is a serine/threonine protein kinase composed of the catalytic subunit as well as a regulatory component, the Ku70/Ku80 heterodimer, which binds DNA ends. DNA-PK plays a role in non-homologous end joining and is essential to the proper functioning of

the mechanisms of DSB repair and V(D)J recombination as well as a number of other processes (for review, see (13)).

Mutations in DNA-PKcs have been associated with SCID in both Arabian foals (14) and Jack Russell terriers (15). However, of the many severe combined immune deficiencies known in humans, none have yet been associated with mutations at the human *PRKDC* locus. The severity of V(D)J recombination defects in animals with DNA-PKcs mutations correlates with the normal enzymatic activity of DNA-PK in the species (15). Genetic DNA-PKcs deficiency causes a severe V(D)J recombination deficit in horses, which have relatively high normal DNA-PK activity. The level of DNA-PK activity in humans is many times higher than that of horses, suggesting that humans may have a profoundly severe phenotype as a result of a genetic DNA-PKcs mutation. It is possible that a germline mutation in *PRKDC* would be lethal (15). DNA-PKcs defects in mice, in which DNA-PK activity is relatively low, cause a less severe V(D)J recombination deficit than in humans or horses. Nonetheless, in addition to being important tools in hematology, cancer and AIDS research, mice homozygous for the *scid* mutation are a valuable models for the study of combined immune deficiency and for investigation of the normal biological role of DNA-PK in non-homologous end joining. Analysis of the differential requirement for DNA-PKcs between species may reveal important insights into the mechanisms of V(D)J recombination and DSB repair.

Nude (*Foxn1*^{*nu*})

The nude (*nu*) mutation was first reported in 1966 as an autosomal recessive mutation that resulted in hairlessness and failure to thrive (16). In 1968, Pantelouris (17)

observed that *nu* mice were congenitally athymic and exhibited leukopenia. Extensive studies in many laboratories have utilized these T-cell-deficient mice to examine the roles of T-cells in host defense against pathogens and in immune responses to various antigens.

The mouse *nu* mutation is within a gene encoding a forkhead/winged helix transcription factor (18). Members of this family of transcription factors are involved in gene regulation during embryological development and cell differentiation (19). The disrupted gene responsible for the *nu* phenotype was originally designated as *whn* (winged-helix-nude), but was renamed *Hfh11* and, more recently, *Foxn1* to comply with unified nomenclature designation for forkhead/winged helix transcription factors (20). A second mutation at the nude locus, named 'streaker' (*nu^{str}*), occurred in an inbred colony of AKR/J mice (21, 22). The phenotype of these mice resembles that of *nu* mice and the symbol for the mutation has been designated *Foxn1^{nu-str}*. Mutations in homologous genes have also been reported in rats (23) and guinea pigs (24). *Foxn1* is expressed specifically in the epithelial cells of the thymus and skin (18). Thus, the T cell deficiency seen in *nu* mice is a result of a defective thymic microenvironment rather than intrinsic defects in T cell progenitors.

Mice carrying the *nu* mutation are valuable tools for cancer research since they serve as experimental hosts for certain human neoplasms (25) (for review, see (26)). However, mice with the *scid* mutation are now recognized as a more appropriate model for studying human tumor biology than *nu* mice. Nude mice lack mature T cells, but they do have functional B cells and circulating immunoglobulin, as well as high levels of NK cell activity, which can prevent the growth of certain tumors. Many tumors, particularly those of hematopoietic origin, grow better in *scid* mice, which lack both B and T cells.

Some solid tumors, however, such as mammary cancers, appear to grow equally well in *nu* and *scid* mice, and thus *nu* mice are still a commonly used model in studies of solid tumor biology and experimental therapy (27, 28).

The *nu* mouse was originally proposed as a model for DiGeorge syndrome in humans, but this mutant lacks certain characteristics of DiGeorge syndrome, such as parathyroid hypoplasia and cardiac anomalies. DiGeorge syndrome has since been found to be due to deletion of a region of human chromosome 22q11, which contains a number of genes that may be responsible for the syndrome (29). Recently, a nonsense mutation in exon 5 of the homologous human gene, termed winged helix nude (*WHN*), was reported in a family in Italy (30, 31). Two sisters presented with a phenotype of impaired T-cell function, alopecia, nail dystrophy and absence of a thymic shadow. This rare condition is an interesting example of how a mutation in a gene not expressed in hematopoietic cells can result in an immunodeficiency disease in humans.

Scurfy (*Foxp3^{sf}*)

The scurfy (*sf*) mutation was first reported in 1959 (32) and further described in the early 1990's (33-35). Scurfy is an X-linked mutation characterized by anemia, thrombocytopenia and a lymphoproliferative disorder that results in splenomegaly, hepatomegaly, and enlarged lymph nodes. These pathologic changes are accompanied by diarrhea, exfoliative dermatitis and runting (33, 34). Hemizygous males have an average lifespan of 24 days (33). The lymphoproliferative disease in *sf* mice is mediated by CD4⁺CD8⁻ T lymphocytes (36, 37), and involves over-expression of a variety of cytokine genes (38, 39).

The *sf* mutation was at one time proposed to be a model for human Wiskott-Aldrich syndrome (WAS) (35), because *sf* and WAS map to homologous regions of the mouse and human X chromosomes (40, 41). However, *sf* has recently been identified as a mutation in the *Foxp3* gene, which encodes a novel forkhead/winged helix transcription factor called scurfin (42). Although the phenotype of *sf* mice clearly demonstrates the requirement of the scurfin protein for normal T cell function, the precise role of this transcription factor and the mechanisms that regulate its expression have not yet been elucidated.

The growing family of forkhead/winged helix transcription factors contains many members that are highly conserved between species (19, 20, 43). The human homologue of the mouse scurfin gene has been identified. Mutations in this gene, *FOXP3*, have been found associated with a human X-linked syndrome of immune dysregulation, polyendocrinopathy and enteropathy called IPEX (44-46) (for review, see (47)). IPEX is a frequently fatal disease of childhood that shares many of the phenotypic characteristics of the *sf* mouse (48). Patients suffer from insulin-dependent diabetes mellitus (IDDM), hypothyroidism, immune thrombocytopenia, and eczema. There are currently no treatments shown to have long-term success in IPEX patients (49). The mouse *sf* mutation provides a valuable model for the study of this severe human autoimmune disease and may assist in the development of immunological therapy. Studies of these mice may also offer further insights into the molecular mechanisms involved in the regulation of T cell function in the normal human immune system.

Lymphoproliferation (*Tnfrsf6^{lpr}*) and generalized lymphoproliferative disease (*Tnfrsf6^{gld}*)

Mice homozygous for the lymphoproliferation (*lpr*) and generalized lymphoproliferative disease (*gld*) mutations share nearly identical phenotypes, characterized by systemic autoimmunity and massive T-lymphoid hyperplasia. Accompanying polyclonal B cell activation results in hypergammaglobulinemia and high levels of autoantibodies against a variety of autoantigens in both *lpr* and *gld* mice, contributing to the development of immune complex glomerulonephritis. Both T- and B-lymphocyte populations of *lpr* and *gld* mice are deficient in their responses to exogenous immune stimuli (50, 51) (for review, see (52)).

The *lpr* mutation arose during the development of the MRL/MpJ inbred strain, and was first reported in 1978 (50, 53) as a model of systemic lupus erythematosus (SLE). MRL wild type mice do not exhibit lymphoproliferation, but do develop SLE-like symptoms at about two years of age, leading to the original hypothesis that the *lpr* mutation may act only to accelerate the development of autoimmune disease in strains already prone to autoimmunity. Although transfer of this mutation to a variety of inbred backgrounds lacking a predisposition to autoimmunity results in lymphadenopathy and autoimmunity, the severity of disease varies depending on the genetic background of the strain (54). MRL/MpJ mice are the most severely affected by the *lpr* mutation; homozygotes die by 6 months of age.

The observation that neonatal thymectomy significantly reduces autoimmune disease and lymphadenopathy in *lpr* mice (55) was followed by the discovery that the *lpr* mutation caused the aberrant expansion of an abnormal population of CD4⁻ CD8⁻ (double negative, DN) T cells (56). Approximately a decade later, the *lpr* mutation was found to

be in the gene encoding the Fas antigen (also called CD95 or APO-1), a widely expressed cell surface protein that mediates apoptosis and is involved in the negative selection of autoreactive T cells in the thymus (57, 58). Fas shares structural homology with the tumor necrosis factor receptor family, and the gene has since been renamed 'Tumor necrosis factor receptor superfamily, member 6' or *Tnfrsf6*. A new allele at the *lpr* locus was reported in 1990, termed *lpr^{cs}* (59), resulting in a phenotype similar to the *lpr* mutation. The *lpr* mutation results in the absence of cell surface Fas, while the *lpr^{cs}* mutation results in expression of a non-functional receptor (60). The *gld* mutation, which produces a phenotype similar to *lpr*, occurred in a colony of C3H/HeJ inbred mice (51, 61). The *gld* mutation was found to be the result of a point mutation in the gene encoding the ligand for Fas (FasL, CD95L) (62), now termed 'tumor necrosis factor superfamily member 6' (*Tnfsf6*).

Fas mediated apoptosis is crucial to the proper development and function of the immune system (see review (63)). Studies of *lpr* and *gld* mice have provided valuable insights into the death receptor mediated system and its role in human disease (for review see (64)). In humans, mutations affecting both Fas (*TNFRSF6*) and Fas ligand (*TNFSF6*) are associated with Autoimmune Lymphoproliferative Syndrome (ALPS), a childhood disease characterized by lymphadenopathy and autoimmunity. Mutations of *TNFRSF6* are commonly associated with a form of ALPS called ALPS1A, also known as Canale-Smith syndrome (65-68). A mutation of *TNFSF6* was discovered in a screening of SLE patients (69). This patient had lymphoproliferative disease as well as symptoms of SLE, and the case was later designated as an alternative form of ALPS, called ALPS1B (70). Somatic mutations of Fas have also been associated with a number of human

malignancies including T-cell leukemia (71, 72), multiple myeloma (73), and non-Hodgkin's lymphoma (74) (for review, see (75)).

Dominant white spotting (*Kit^w*) and steel (*Kitl^{sl}*)

Mutations at the dominant white spotting (*W*) (76, 77) and steel (*Sl*) (78) loci affect the development of hematopoietic precursor cells, mast cells, neural crest-derived melanocytes and germ cells. Consequences of these developmental abnormalities include macrocytic anemia, mast cell deficiency, pigmentation abnormalities and infertility (reviewed in (79)). There are a number of alleles at both the *W* and *Sl* loci, resulting in diverse phenotypic effects that vary in severity from embryonic lethality to mild anemia with survival to adulthood (79-81)

Although the phenotypes of *W* and *Sl* mutant mice are similar, numerous early studies indicated that the *sl* mutation affected the extracellular microenvironment, while the *W* mutation was within a gene expressed by the affected stem cells themselves (82-86). The genes disrupted by these two mutations were found to encode a growth factor/receptor pair essential to a variety of developmental processes. The *W* mutation affects the *Kit* proto-oncogene (also known as *c-Kit*), which encodes a transmembrane receptor tyrosine kinase, KIT. (87, 88). The steel mutation affects the gene encoding stem cell factor (SCF) (also called mast cell growth factor, kit-ligand or steel factor, current approved gene symbol: *Kitl*), which is the ligand for KIT (89-92) (see review (93)).

KIT is expressed at various levels on a wide variety of cell types. SCF (*Kitl*) is expressed on stromal cells, fibroblasts and endothelial cells, and is found at low levels in

the circulation. This growth factor/receptor system has been implicated in a wide variety of processes, from promotion of hematopoietic cell survival and proliferation to mediation of cell migration and adhesion (for review, see (94-97)).

In humans, heritable mutations in the homologue of the mouse *Kit* gene, *KIT*, have been found to cause piebaldism (98, 99), a genetic disorder resulting in patches of white skin and hair due to a disorder in melanocyte development. In contrast to the *W* mouse, individuals affected by piebaldism exhibit no apparent defects in mast cell function nor do they develop anemia as a consequence of the *KIT* mutation. Spontaneous, non-familial mutations in the *KIT* gene have been found in cases of human mast cell neoplasia and cutaneous mastocytosis (100, 101), as well as in tumors of the gastrointestinal stroma (102, 103). These mutations often cause constitutive activation of *KIT* (for a review of *KIT* activating mutations, see (104)). No mutations have been found in *KITLG*, the human homologue of the mouse stem cell factor gene.

X-linked immune deficiency (*Btk*^{*xid*})

X-linked agammaglobulinemia (XLA) in humans was first reported as a primary immunodeficiency disease in 1959 (105). XLA patients have severely decreased levels of mature B lymphocytes, dramatically reduced levels of serum immunoglobulin (Ig) and are highly susceptible to bacterial infections (105, 106). Many years later, the X-linked immune deficiency (*xid*) mutation was observed in the CBA/N mouse strain, resulting in a disease with characteristics similar to, but less severe than human XLA (107-111). Peripheral B cells in *xid* mice are moderately reduced in number, and those present display a primarily immature phenotype. Mouse *xid* is also characterized by impaired

signaling through a variety of B-cell surface receptors, absence of peritoneal B-1 cells, reduced levels of serum IgM and IgG3 and failure to respond to type II thymus-independent (TI-II) antigens (reviewed in (112)).

The mutations causing both the human and mouse diseases lie within the respective genes encoding a cytoplasmic protein-tyrosine kinase, coined Bruton's agammaglobulinemia tyrosine kinase (Btk) (113-116). *Btk* is a member of a growing family of homologous tyrosine kinases (reviewed in (117, 118)). Although *Btk* appears to function primarily in the B-cell lineage, in development and expansion of early B cells and in survival and activation of mature B cells, this tyrosine kinase is expressed in erythroid progenitors and monomyeloid cells in addition to B cells (113, 114). *Btk* is not, however, expressed on T or NK cells. Mouse *xid* results from a specific missense mutation in *Btk* affecting the pleckstrin homology (PH) domain of the BTK protein (115, 116).

Human XLA has been found associated with an extensive variety of *BTK* mutations in affected individuals (119). A comprehensive database of these mutations has been established to assist clinicians and researchers in their study of the disease (120). Efforts have been made to relate the specific BTK domain mutated to the severity of the phenotype in humans but no definitive connections have been drawn. The presence of phenotypic variation in XLA, even among affected individuals with identical genetic abnormalities, highlights the existence of modifier genes affecting B cell development. Modifier genes, or some differential requirement for the BTK protein between species, is the likely cause of the disparity of disease severity between mouse and human, as well. Human *BTK* mutations that are almost identical to mouse *xid* do not

result in a milder XLA phenotype than other *BTK* mutations. Moreover, mice with a targeted null mutation of *Btk* have a phenotype similar to that of *xid* mice (121, 122), reaffirming that absence of *BTK* function, not the nature of the mutation, results in the species specific B-cell defects seen in XLA patients and *xid* mice. The *BTK* gene is involved in a wide array of complex functions. Studies of the disease variations between mouse and human and among individual human XLA patients have contributed to the elucidation of the role of *BTK* in B cell development and signaling and revealed insights into other pathways involved in B cell function. These discoveries have been the subject of a number of recent reviews (123-126). New techniques of identifying *BTK* mutations in humans have also led to the identification of several other genetic B lineage defects (127).

Alymphoplasia (*Map3k14^{aly}*)

Alymphoplasia (*aly*) mutant mice are homozygous for an autosomal recessive mutation resulting in absence of lymph nodes and Peyer's patches, structural defects in the spleen, and impaired antibody and cell-mediated immune responses (128-130). Recent studies have shown that in addition to structural defects in secondary immune organs, *aly* mice have intrinsic defects in both B and T cells (131, 132). Thus, the abnormal immune function seen in these mice appears to be due to both stromal insufficiency and an innate defect in lymphocyte function.

Recently, Nuclear Factor (NF)- κ -B-inducing kinase (*Nik*) was identified as the mutated gene responsible for the *aly* mutation (133). A member of the mitogen activated protein kinase family (134), the *Nik* gene has since been renamed Map kinase kinase

kinase 14 (*Map3k14*) (81) to comply with evolving standard nomenclature. The NIK protein is a serine/threonine kinase involved in the induction of NF- κ -B through a number of different cytokine receptors (134). The mutation in *aly* mice encodes a defect within the carboxyl-terminal interacting domain of NIK, disrupting its ability to associate with the adaptor proteins that target its activity, while leaving its kinase region intact (133).

The mechanisms through which NIK functions in cytokine signaling and how the functional NIK deficit seen in *aly* mice could result in their characteristic phenotypic defects are unclear. While it is widely accepted that NIK stimulates NF- κ -B activity, the receptors that the kinase conveys signals through have not been fully clarified (134-137). NF- κ -B is a transcription factor that is induced by many cytokines and regulates the expression of a large number of immune and inflammatory genes (138, 139). In the original report of the discovery of the NIK protein, NIK was implicated in NF- κ -B induction through TNF receptors, CD95 and IL-1 (134). A recent study using mice carrying a targeted deletion of the NIK gene contradicts these findings and implicates NIK in regulation of signaling through the lymphotoxin- β receptor (137). Although the NIK deficient mice used in this latter study have a complete absence of NIK protein whereas *aly* mice produce normal levels of a mutated NIK protein, their phenotypes are almost identical.

The human *NIK* gene was considered an interesting positional and biological candidate for frontotemporal dementia with parkinsonism linked to Chromosome 17 (FTDP-17) (OMIM 600274) (140). However, mutational screening has not revealed concomitant mutations of the *NIK* gene with FTDP-17 (140). Mutations in the gene

encoding microtubule-associated protein tau (*MAPT*) have since been found associated with some cases of FTDP-17 (141). There are currently no known human diseases caused by mutations in the *NIK* gene. However, X-linked anhidrotic ectodermal dysplasia with immune deficiency (EDA-ID) (OMIM 300291) has recently been reported to be associated with mutations in the gene *IKBKG* (142), which encodes a protein (IKK- γ , NEMO, FIP-3) that interacts with the NIK protein (143) and is involved in regulation of NF- κ -B activation. Mutations in *IKBKG* cause impairment of NF- κ -B signaling which results in EDA-ID. In addition, a number of inflammatory diseases including rheumatoid arthritis, asthma and multiple sclerosis are associated with enhanced NF- κ -B induction. Studies of *aly* mice will continue to contribute to the understanding of the complex signaling pathways involved in NF- κ -B induction in hematopoietic cells. Clarification of these pathways will provide further insight into the inflammatory process, assisting in the development of novel therapies for a diverse range of inflammatory diseases.

Beige (*Lysf^{bg}*)

The beige (*bg*) mutation was first identified as a coat color mutation that resulted in large, abnormal granules in all granule containing cells, including granulocytes and melanocytes (144, 145). In addition to abnormal hair pigmentation, *bg* mice have defective cytotoxic T cell responses and a deficiency in NK cell function. Beige mice display increased susceptibility to infection by a number of pathogens, including cytomegalovirus (146) and *Mycobacterium avium* (147). These mice also exhibit a reduced ability to reject transplanted tumors and have a bleeding disorder due to a platelet storage pool deficiency (148). Characteristic features of the *bg* mutation are also found

in Chediak-Higashi Syndrome (CHS) in humans (145, 149) and in a similar disease described in a number of other mammals, including mink, cattle, cats and killer whales (150). There are also a number of different alleles of *bg* resulting from distinct spontaneous mutations in the mouse (81).

Complementation analysis had suggested that the same gene was responsible for mouse *bg*, human CHS and CHS-like disease in other species (151). Two groups independently identified the mouse *bg* gene (152, 153), which encodes a widely expressed cytosolic protein implicated in the regulation of lysosomal fission (154) and has been designated the lysosomal trafficking regulator gene (*Lyst*). Identification of the mouse *bg* gene led to the discovery of a novel human gene with significant sequence homology to the mouse gene (153, 155). Sequencing of the human gene, currently called *CHS1*, revealed the presence of mutations in several CHS patients (153, 155). Subsequent studies confirmed that the homologous gene was responsible for the CHS-like disease in rats (156) and cows (157). The precise function of the CHS/beige protein is not yet clearly understood. Two recent reviews contain useful background information and summaries of the current knowledge in the field (148, 158).

The disease phenotype seen in *bg* mice and human CHS patients is remarkably similar. However, CHS patients commonly develop a lymphoproliferative disorder termed the 'accelerated phase' of the disease, which does not occur in *bg* mice. It has recently been reported that intracellular trafficking of CTLA-4 (CD152) is defective in CHS patients (159). CTLA-4 is a negative regulator of T cell activation, and the decreased expression of this molecule on T cells of CHS patients may disrupt T cell homeostasis resulting in the lymphoproliferative syndrome. CTLA-4 trafficking in *bg*

mice is relatively unaffected. Therefore, the absence of the lymphoproliferative disease in *bg* mice may be a function of species-specific variations in intracellular processing of the CTLA-4 protein.

Osteopetrosis (*Csf1^{op}*)

Mice homozygous for the osteopetrosis (*op*) mutation are small in size and toothless, with a shortened lifespan and reduced fecundity. They have a severe osteoclast deficiency and exhibit defects in bone remodeling (160). The *op* mutation affects the development and differentiation of most macrophage subpopulations, resulting in reduced numbers of osteoclasts as well as tissue macrophages in *op* mice (161), functional deficits in peritoneal macrophage populations (162, 163) (reviewed in (164)) and phenotypic abnormalities in dendritic cells (165). Due to reduction of marrow cavity volume, bone marrow hematopoiesis in *op* mice is severely reduced (162), and compensatory extramedullary hematopoiesis is seen in both spleen and liver (166).

Transplantation of bone marrow or spleen cells from wild type mice does not rescue the osteopetrotic phenotype of *op* mice (167). This observation suggested that the *op* defect was in the stromal microenvironment rather than the hematopoietic stem cells themselves, and led to the discovery that the *op* mutation is a single base change in the macrophage colony stimulating factor gene, *Csf1* (168). This gene encodes macrophage colony stimulating factor (CSF-1, also called M-CSF), which is a cytokine involved in regulating osteoclastogenesis and macrophage production. CSF-1 also plays a role in brain development (169) and in both male and female reproduction (for reviews, see (170, 171)).

Of the many forms of osteopetrosis in humans, none has yet been proven associated with mutations in the human *CSF1* gene. An autosomal dominant form of osteopetrosis showed linkage to the chromosomal region in which human *CSF1* is located (172), but mapping data did not confirm linkage with the *CSF1* gene (173). Nonetheless, *op* mice and other osteopetrotic mutants have been very valuable in studying normal bone biology as well as the pathophysiology of bone resorption disease in humans (for review, see (174)). Much of the current knowledge of the physiological functions of CSF-1 has been discovered through the study of osteopetrotic mice.

Defective lipopolysaccharide response (*Tlr4^{lps-d}*)

Bacterial endotoxin is a component of the cell wall of Gram-negative bacteria. Also known as lipopolysaccharide (LPS), endotoxin elicits a potent inflammatory response in most strains of mice as well as in many other mammalian species. More than 30 years ago, it was discovered that C3H/HeJ mice exhibited a defective immune response to lipopolysaccharide (175-177), providing a model to study the physiology of the endotoxin response as well as the genetic control of this response (178). The defective allele for the endotoxin response was labeled *Lps^d*, to distinguish from the normal allele, *Lpsⁿ*.

Endotoxin exerts its effect on multiple cell types in the immune system (179). The normal endotoxin response includes mitogenic stimulation of B cells (180, 181), proliferation of a subpopulation of T cells (182), macrophage cytotoxicity (183) and inhibition of the phagocytic capacity of macrophages (184). C3H/HeJ mice are defective in these normal cellular responses to LPS. They do not produce the high levels of pro-

inflammatory cytokines that normally characterize the LPS response, and are therefore more resistant to the lethal effects of LPS (185). As a result, however, C3H/HeJ mice are more susceptible to infection by Gram-negative organisms (186, 187). In 1977, it was reported that C57BL/10Cr mice also exhibit a defective LPS response (188), which was mapped to the same locus disrupted in the C3H/HeJ strain (189, 190).

The mutations responsible for defective LPS responses in mice were recently found to disrupt the Toll-like receptor-4 (*Tlr4*) gene (191, 192). Toll-like receptors play a key role in the induction of the innate immune response, which is the first line of defense against infectious organisms (for review, see (193, 194)). The family of mammalian Toll-like receptors have significant homology to *Drosophila Toll* (195) (for review, see (196)). TLR4 is a transmembrane protein that conveys signals which activate NF- κ B to promote expression of the genes involved in the LPS response (197). TLR4 appears to function only in the LPS response pathway, so other immunological functions are normal in *Lps^d* mice. Mice with a targeted mutation of the *Tlr4* gene have a phenotype that very closely resembles that of C3H/HeJ mice carrying the *Lps^d* allele (198). While both C3H/HeJ and C57BL/10Cr mice have mutations in *Tlr4*, differences had been observed in levels of interferon (IFN)- γ production between the two strains in response to various microorganisms (199). This variation has recently been recognized as the result of a second mutation in the C57BL/10Cr strain, in the *IL-12R β 2* gene (200), which results in deficient induction of IFN- γ through the IL-12 receptor (201). Thus, C57BL/10Cr mice are even more resistant to the toxic effects of LPS than C3H/HeJ mice.

In humans, exposure to inhaled endotoxin can cause various degrees of airway inflammation and asthma (202). The observation that certain individuals are sensitive to

environmental endotoxin exposure while others are more resistant prompted the examination of the genetic regulation of this response in humans. Screening of a group of subjects for both *TLR4* mutations and degree of responsiveness to inhaled LPS revealed that mutations in the human *TLR4* gene were associated with airway hyporesponsiveness to LPS challenge (203). Like the C3H/HeJ mouse, humans with reduced endotoxin responsiveness may be more susceptible to infection by Gram-negative organisms. Further examination of the role of *TLR4* in LPS signaling may disclose new approaches in the treatment of endotoxin mediated diseases.

Other mutations {microphthalmia (*Mitf^{mi}*), wasted (*Eef1a2^{wst}*), hairless (*hr*)}

A number of other spontaneous mouse mutations have immunological abnormalities as a component of a broader phenotype. One of these is microphthalmia (*mi*) (204). Mutations at this locus affect pigmentation, secondary bone resorption, eye development, hearing and immunological function (reviewed in (205)). The immunological changes include reduced numbers and deficient function of mast cells and NK cells (206) and osteoclast defects resulting in osteopetrosis (207). The *mi* gene has been identified as a transcription factor in the basic-helix-loop-helix-leucine zipper (bHLH-ZIP) family (208, 209), now named *Mitf*, which regulates transcription through binding to specialized motifs found in melanocyte specific promoters. Recent studies suggest that the immunological defects may be due both to a defect in the bone marrow microenvironment (210) and to intrinsic cellular abnormalities (211, 212). See (213, 214) for reviews of the role of MITF protein in melanocyte signaling and development. Mutations in human *MITF* have been found associated with Waardenburg syndrome

(WS) type II (215, 216). WS type I (OMIM 193500) and type III (OMIM 148820) are caused by mutations in the *PAX3* gene (217) which has been shown to regulate *MITF* gene expression (218).

Mice homozygous for the wasted (*wst*) mutation were noted for their neurological phenotype. Characterized by development of tremors and ataxia beginning at about 21 days of age, followed by weight loss, progressive paralysis and lymphoid hypoplasia, *wst* mice die shortly after weaning (219). The immunological abnormalities of *wst* mice include increased sensitivity of hematopoietic cells to the cytotoxic effects of ionizing radiation (219, 220), defects in secretory Ig responses (221, 222), disproportionate T-cell subsets with abnormalities in cytokine levels (223) and sensitivity of thymocytes to apoptosis (224). The *wst* mutation is caused by a deletion in *Eef1a2* gene encoding an isoform of eukaryotic translation elongation factor 1 α (EF1 α) (225). *Eef1a2* is expressed only in brain, heart and skeletal muscle, and plays a role in protein synthesis in mammalian cells (226)(for review, see (227)). The neurological phenotype in *wst* mice is presumably related to defects in protein translation in the absence of EF1 α 2 in brain, heart and skeletal muscle of these mice. However, questions still remain as to how loss of this protein can cause the immunological deficits seen in *wst* mice, since the protein has not been detected in splenic tissue of either mice or rats (225). Although *wst* was originally proposed to be a model of ataxia telangiectasia (AT) (219, 228, 229), AT is now known to be associated with mutations in the human *ATM* gene (230). The human homologue of the *wst* gene, *EEF1A2*, has been identified and sequenced (231), but there is currently no known disease caused by mutations of this gene in humans.

The most apparent feature of the hairless (*hr*) mutation is total loss of hair beginning at 3 – 4 weeks of age (232-234). In addition to alopecia, *hr* mice also have age related immunological defects and a high incidence of thymic lymphoma (235-237) (for review, see (238, 239)). There are a number of different *hr* alleles (81). The wild type gene encodes a predicted 1182 amino acid protein, 'HR', containing a zinc finger domain with a 4-cysteine motif (240). The *hr* gene is widely expressed, and it has been speculated that the HR protein may act as a transcription factor (240); however, its true functions have not yet been resolved. Although thymic defects have been reported in *hr* mice, no evidence of *hr* protein expression has been detected in the thymus (241). Mutations of the human *HR* gene have been found in individuals with congenital atrechia with papules (242-245). This disorder is characterized by complete hair loss and papular lesions similar to those seen in mice homozygous for the rhino allele of hairless (*hr^{rh}*); however, immunological abnormalities have not been reported in these patients. The molecular basis of the hairless mutations and currently known functions of the *hr* gene in both humans and mice have been reviewed recently (246-248).

Motheaten (*Hcph^{me}*)

The original motheaten (*me*) mutation occurred spontaneously on the inbred C57BL/6J background (249). Homozygous *me* mice are severely immunodeficient, express high levels of serum autoantibodies (250-253) and die at approximately 3 weeks of age from inflammatory lesions in the lungs and elsewhere. A second mutation at this locus, 'viable motheaten' (*me^v*), was reported in 1984 (250). Homozygous *me^v* mice have a similar but less severe phenotype, with a lifespan of approximately 9 weeks. The

longer lifespan of *me^v* mice in comparison to *me* mice allowed more extensive study of the nature of the mutation.

In 1993, the motheaten mutations were determined to be in the hematopoietic cell phosphatase (*Hcph*) gene (254, 255). The *Hcph* gene encodes the Src homology region 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1) (256, 257), which functions as a negative regulator of signaling through a number of hematopoietic growth factor receptors. SHP-1 exerts its negative regulatory role in hematopoietic cell growth, differentiation and activation by dephosphorylating receptors, such as CD72, receptor-associated tyrosine kinases such as ZAP-70 or Jak2, and other signaling molecules recruited through adaptor proteins such as Grb-2. (For a thorough review of SHP-1 signaling see (258)). SHP-1 has also recently been found to play a critical role in the induction of apoptosis and cell cycle arrest in response to γ -irradiation (259). The phenotypic differences between the two allelic mutations at the *me* locus are due to a variation in expression levels of the SHP-1 protein. The *me* mutation results in the total absence of SHP-1 protein due to creation of a premature termination codon, while the *me^v* mutation (*Hcph^{mev}*) results in a significant reduction in SHP-1 activity due to the generation of several alternative splicing sites.

Although the motheaten mutations are not a model for any known human disease, they provide a valuable resource for the study of the role of a specific protein tyrosine phosphatase (PTP) in the development and regulation of the immune and hematopoietic systems. Studies of motheaten mice have provided insight not just into the function of the SHP-1 phosphatase but into the general function of phosphatases and their integral role in regulating an enormous variety of cell signaling cascades and cell functions (258).

The human homologue of the mouse *Hcph* gene is called *PTPN6* (260). No known heritable mutations exist in human *PTPN6*. If germ-line mutations in this gene do occur in humans, they may well result in embryonic lethality. Recently, however, aberrant SHP-1 expression has been associated with a variety of human cancers. An errant splice product of *PTPN6* has been found in human myeloid leukemia cells (261). This post-transcriptional processing error is thought to result in a functional haplo-insufficiency of SHP-1 protein, possibly contributing to leukemogenesis (261). SHP-1 expression is absent or significantly reduced in many human T-cell lymphomas as a result of a transcriptional block caused by methylation of the SHP-1 promoter (262). The absence of SHP-1 protein is associated with constitutive activation of the interleukin 2-receptor (IL-2R) -associated Jak/STAT pathway and may contribute to the malignant transformation human T cells (262). Defective expression of SHP-1 has also been implicated in the pathogenesis of polycythemia vera (263), and up-regulation of SHP-1 expression has been found associated with human breast cancers (264).

Conclusion

Determination of the molecular bases of mouse immunological mutations has increased the understanding of the mechanisms of disease and aided in elucidating the roles of these genes in the intact immune system. Certain of these immunological mutations serve as homologues for human immunodeficiency and autoimmune diseases (70, 81, 265). Although the pathological changes related to these mutations may be affected by physiological differences between mouse and man, the mouse models provide an invaluable resource for biomedical researchers. Continued studies of new

immunological mutations at The Jackson Laboratory and elsewhere will likely lead to further insights into the fundamental aspects of immune defense and expand the knowledge of the immune system.

Chapter 2

SHP-1 PROTEIN TYROSINE PHOSPHATASE AND THE “MOTHEATEN” MOUSE

Tyrosyl phosphorylation is a key regulatory mechanism involved in development and function of the immune system. The intricate dance between tyrosine kinases and phosphatases is crucial for maintaining balance in hematopoietic signaling. One misstep can have severe consequences, resulting in autoimmunity, immune deficiency, or cancer. As a result of the development of gene targeting techniques, there are now many mouse models lacking specific components involved in regulation of immune function. However, one of the first models for a specific tyrosine phosphatase deficiency was found in mice with one of two spontaneous allelic mutations at the motheaten locus, introduced in Chapter 1 of this manuscript.

As discussed in Chapter 1, mice carrying mutations at the motheaten locus are deficient in SHP-1 (249, 250, 254). SHP-1 is a cytosolic protein tyrosine phosphatase (PTP) that plays a crucial role in regulating proliferation, differentiation, and other signaling processes in cells of the immune and hematopoietic systems. (For a review of PTP's in signal transduction, see (266)). As a result of SHP-1 deficiency, mice homozygous for the *me* or *me*^v mutations have multiple hematological and immunological defects that are discussed in detail elsewhere. In addition to being valuable tools for the elucidation of SHP-1 function, studies using *me* and *me*^v mice have provided many insights into a wide range of signaling pathways and mechanisms essential to maintenance of immune homeostasis (267).

SHP-1 functions primarily in the negative regulation of hematopoietic signaling events induced by cytokines, growth factors and antigens (for review, see (258)). The 68 kD SHP-1 protein, (previously known as PTP-1C, HCP, SHP, SHPTP1 and PTPN6), contains two N-terminal Src-homology 2 (SH2) domains, a phosphatase catalytic region, and a C-terminal region compatible with tyrosine phosphorylation (257, 267) (Figure 2.1). The SH2 domains mediate the recruitment of the SHP-1 protein to phosphorylated tyrosine residues within immunoreceptor tyrosine-based inhibitory motifs (ITIM's) in the cytoplasmic regions of inhibitory receptors and co-receptors (see review (268)). Structural rearrangement of the SHP-1 protein as a result of SH2 domain binding activates the phosphatase catalytic domain (269), which dephosphorylates the intended substrate, resulting in signal inhibition.

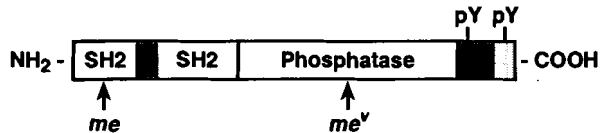


Figure 2.1. Structure of SHP-1, showing the locations of the *me* and *me'* mutations.

SHP-1 has been shown to interact with a variety of both cytoplasmic and membrane bound molecules in the regulation of B-cell signaling, including Lyn kinase, CD22 (270), paired immunoglobulin-like receptor B (PIR-B) (271) and CD72 (272) (Figure 2.2). The SHP-1 phosphatase is also involved in regulation of T cell receptor (TCR) signaling function (273) (Figure 2.3). SHP-1 associates with CD5, which is a transmembrane protein expressed on all T cells, and the B-1a subset of B cells (274).

Activated B cells

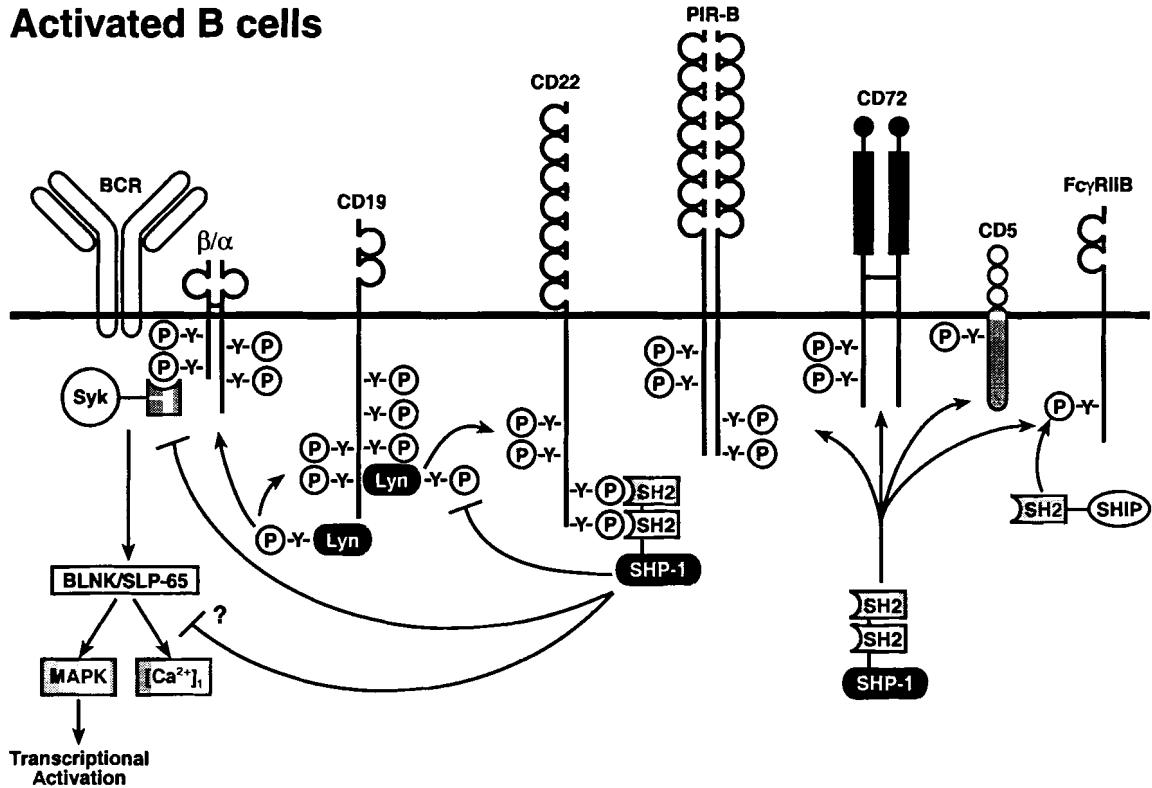


Figure 2.2. Functions of SHP-1 in regulating signals through the B-cell receptor (BCR). After BCR cross linking, the Src-family protein tyrosine kinase (PTK) Lyn has the principal role in phosphorylation of tyrosine residues in both the immunoreceptor tyrosine-based activation motifs (ITAMs) of Ig- α/β , as well as in the ITIMs of inhibitory co-receptors (such as CD22, PIR/B, CD72 and Fc γ RIIB). Phosphorylation of ITIMs by Lyn results in recruitment of SHP-1 through its SH2 domains. From its position at the cell membrane, SHP-1 dephosphorylates activated PTK's and, by extension, PTK substrates, resulting in inhibition of BCR mediated signals. (Figure based on (258)).

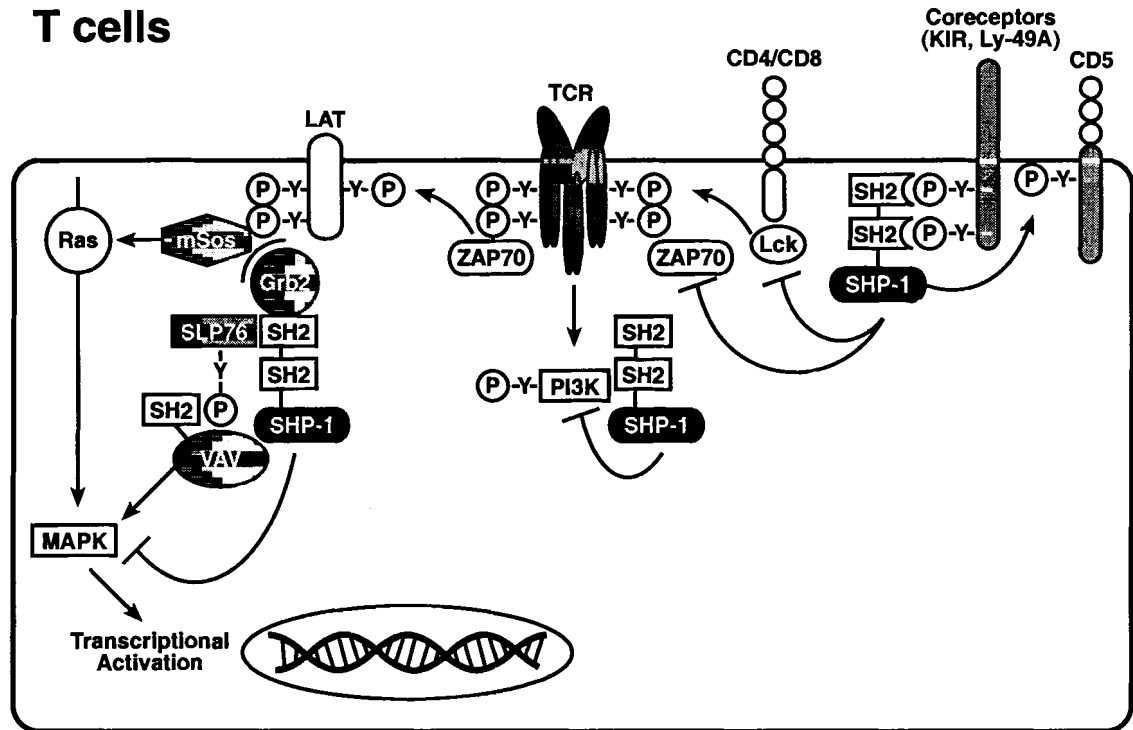


Figure 2.3. Functions of SHP-1 in regulating signals through the T-cell receptor (TCR). TCR engagement induces the transphosphorylation of the Syk-family PTK ZAP-70 by the Src-family PTK Lck, which is associated with the CD4/CD8 co-receptor. The consequent phosphorylation of ITIMs within inhibitory receptors such as KIR, Ly-49A and CD5 results in recruitment of SHP-1 through its SH2 domains. SHP-1 can directly associate with and dephosphorylate receptors or activated PTKs, or indirectly dephosphorylate other signaling effectors such as SLP-76, through association with adaptor proteins such as Grb2. (Figure based on (258)).

B-1a B cells are often associated with autoimmunity in humans (reviewed in (275)), and constitute a large percentage of total B cells in *me^v/me^v* mice. Chapter 3 describes studies using mice homozygous for both the *me^v* mutation, and a targeted deletion of the CD5 gene. These mice were developed to investigate the function of CD5-mediated signaling in development of autoimmunity in the absence of SHP-1, as well as to provide insight into the role of SHP-1 in regulating signals through the CD5 receptor.

Cells of the myeloid lineage are also dependent on SHP-1 to regulate signals through a multiplicity of receptors. In particular, macrophages from motheaten mice are hyper-responsive to signals through receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) (276) and macrophage colony stimulating factor (M-CSF or CSF-1) (277), which direct proliferation and survival of cells of the granulocyte-macrophage lineage and their progenitors. Heightened proliferation and accumulation of myeloid-lineage cells is a characteristic of the motheaten mouse. Chapter 4 describes the generation of mice transgenic for macrophage specific expression of a dominant negative form of the SHP-1 protein, to investigate the cell autonomous effects of absence of functional SHP-1 in macrophages and the effects of dysregulated macrophages on other cell populations.

With the known role of SHP-1 as a negative regulator of cell signaling, it is logical to postulate that loss of function of this regulatory protein could contribute to malignant transformation, or that SHP-1 can act as a tumor suppressor gene. The association of SHP-1 mutations with human cancers is discussed in the section on the “motheaten” mouse in Chapter 1 (261, 262). Studies are ongoing in our lab to identify neoplasms arising in aged *+/me* and *+/me^v* mice caused by loss of SHP-1 heterozygosity,

in order to substantiate the putative role of SHP-1 as a tumor suppressor gene (254).

Chapter 5 describes an osteogenic cell line derived from a spontaneous osteosarcoma that arose in a 25-month old C57BL/6J $+/-me^v$ mouse. Preliminary studies of this malignant neoplasm revealed that it was not caused by loss of expression of the SHP-1 protein.

However, the cell line by itself, or when used in transplantation experiments, has value as a model for studying human osteosarcomas.

Several other molecules exist which share some structural homology with SHP-1, including SHP-2 (278) and an SH2 containing inositol polyphosphate 5'-phosphatase, SHIP (279), both of which contain SH2 domains and may serve purposes similar to SHP-1 in some incidences. However, it is evident by the severity of disease caused by SHP-1 deficiency that there are few, if any, precisely redundant molecules that may serve as a substitute for SHP-1 in living systems. The known biological roles of SHP-1 are multitudinous, yet many of its functions still remain a mystery. Further investigation into the functions of this phosphatase may provide clues to expand the knowledge of a great many signaling pathways involved in normal immunological function.

Chapter 3

ABSENCE OF CD5 DRAMATICALLY REDUCES PROGRESSION OF PULMONARY INFLAMMATORY LESIONS IN SHP-1 PROTEIN-TYROSINE PHOSPHATASE-DEFICIENT “VIALE MOTHEATEN” MICE ²

Abstract

Mice homozygous for the viable motheaten (*Hcph^{me-v}*) mutation are deficient in SHP-1 protein-tyrosine phosphatase, resulting in severe systemic autoimmunity and immune dysfunction. A high percentage of B-cells in viable motheaten mice express the cell surface glycoprotein CD5, in contrast to wild type mice that express CD5 on only a small percentage of B-cells. CD5⁺ B-cells have been associated with autoantibody production. To determine the role of CD5 in the development of the inflammatory disease in *me^v/me^v* mice, we created a stock of *CD5^{-/-}me^v/me^v* mice. The longevity of *CD5^{-/-}me^v/me^v* mice was increased 69% in comparison to *me^v/me^v* mice on a similar B6;129 background. The increased lifespan was associated with a marked reduction in pulmonary inflammation. Flow cytometry analysis of spleen cells from *CD5^{-/-}me^v/me^v* mice at 9 – 12 wks of age revealed significant decreases in percentages of IgM/B220 double positive B-cells, Mac-1/Gr-1 double positive cells and CD4⁺ T-cells compared with *me^v/me^v* mice. *CD5^{-/-}me^v/me^v* mice also had significantly lower serum IgM levels in comparison to *me^v/me^v* mice. Study of *CD5^{-/-}me^v/me^v* mice may provide further insight

² Modified from: Joliat MJ, Lang PA, Lyons BL, Lynes MA, Yi T, Sundberg JP, Shultz LD. Absence of CD5 dramatically reduces progression of pulmonary inflammatory lesions in SHP-1 protein-tyrosine phosphatase-deficient viable motheaten mice. *J. Autoimmun.* In press.

into the role of CD5 in cell signaling and may help explain the observed association of CD5⁺ B-cells with autoimmune disease.

Introduction

Mice homozygous for either of the two recessive allelic mutations in the hematopoietic cell phosphatase (*Hcph*) gene on Chromosome 6 are severely immunodeficient, express high levels of serum autoantibodies (250-253) and die at an early age from inflammatory lesions in the lungs and elsewhere. The *Hcph* gene encodes the Src homology region 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1) (256, 257), which is a negative regulator of signaling through a number of hematopoietic growth factor receptors (258, 276, 280). The two mutant alleles of the *Hcph* gene that arose spontaneously in C57BL/6J mice are 'motheaten', (*Hcph^{me}*, abbreviated *me*) and viable motheaten (*Hcph^{me-v}*, abbreviated *me^v*). The *me* mutation is due to a cytosine deletion that results in the total absence of the SHP-1 protein due to creation of a termination codon. Death of *me/me* mice occurs at approximately 3 wks of age. The *me^v* mutation is a thymine to adenine transversion that results in creation of alternative splicing sites and an 80% to 90% reduction in SHP-1 activity compared with wild type mice. Homozygous *me^v/me^v* mice live to approximately 9 wks of age and are thus the more commonly used model to study the consequences of deficiency in SHP-1 protein tyrosine phosphatase (254).

In addition to autoimmunity and severe pulmonary disease, *me^v/me^v* mice have many additional cellular abnormalities, including an increase in myelopoiesis (250, 281) and multiple defects in lymphocyte development and function (282-285). A large

percentage of B-cells present in *me^v/me^v* mice express CD5 (286), which is normally found only on T-cells and a small percentage of B-cells in wild type mice.

CD5 (Ly-1) is a monomeric 67-kD membrane glycoprotein expressed on all mature T-cells and on a subset of B-cells, termed B-1a cells. Classified structurally as a member of the scavenger receptor family (287), CD5 consists of a cysteine rich extracellular region and a cytoplasmic domain containing motifs compatible with phosphorylation by tyrosine and serine/threonine kinases (Figure 3.1). CD5 is part of the TCR complex. It is expressed at low levels on immature, CD4⁺CD8⁺ thymocytes and is upregulated in differentiated T-cells (288). Tyrosine residues within the CD5 cytoplasmic region are phosphorylated upon TCR stimulation (289). Recently, it was reported that CD5 functions as a negative regulator of signal transduction through the TCR, affecting thymocyte differentiation as well as mediating TCR signaling in mature cells (290-292). CD5 has been found to be constitutively associated with SHP-1 in Jurkat T-cells and normal phytohemagglutinin-expanded T-lymphoblasts (274), indicating that the negative regulatory role of CD5 in T-cells may be mediated through SHP-1.

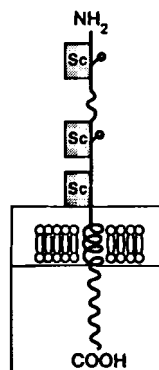


Figure 3.1. Structure of CD5. The extracellular region contains three scavenger receptor cysteine-rich domains, while the cytoplasmic region contains motifs compatible with Ser/Thr or Tyr phosphorylation.

CD5 is associated with the B-cell receptor (BCR) on B-1a cells (293). In wild type adult mice, cells expressing CD5 constitute a significant percentage of the B-cell population in the peritoneal and pleural cavities, while they are scarcely found in the splenic B-cell pool (294). Although the function of CD5 on B-cells is poorly understood, B-1a cells, that express CD5, are associated with production of natural autoantibodies (295), specifically of the IgM isotype (296). An increase in the number of B-cells expressing CD5 is often associated with increased production of autoantibodies (reviewed in (275)). In *me/me* and *me^v/me^v* mice, virtually all of the B-lymphocytes, including the splenic populations, express CD5 (297). We theorized that CD5 deletion in *me^v/me^v* mice would result in reduction of sytemic autoimmunity in these mice.

CD5 deletion in otherwise wild type mice does not interfere with the ability of these mice to mount an efficient immune response (294). T-cells from CD5^{-/-} mice are

hyperreactive in response to TCR mediated signals *in vitro* (290), but this increased reactivity has no apparent detrimental effect on T-cell function *in vivo*. Thus, mice lacking CD5 are healthy and have normal distribution of lymphoid and myeloid cells. To explore the effect that CD5 expression has on the autoantibody production and inflammatory disease of viable motheaten mice, we crossed 129-*Cd5^{tm1Cgn}* (*CD5^{-/-}*) mice with C57BL/6J-*me^v* mice to create a stock of mice homozygous for the CD5 deletion and segregating for the viable motheaten mutation. Comparison of the phenotypes of *me^v/me^v* mice with or without CD5 revealed a marked role of this molecule in the development of immunopathologic changes in *me^v/me^v* mice.

Materials and Methods

Mice

All mice were raised at the Jackson Laboratory (Bar Harbor, ME). 129-*Cd5^{tm1Cgn}* (*CD5^{-/-}*) mice (294) were first crossed with heterozygous C57BL/6J-+/+*me^v* mice. Offspring were heterozygous for *CD5^{-/-}* and were typed for the presence of the *me^v* mutation. Mice that were heterozygous for both the *me^v* mutation and *CD5^{-/-}* were intercrossed. Homozygous *CD5^{-/-}* mice from these matings that were heterozygous for the *me^v* mutation were then intercrossed to create a B6;129-*CD5^{-/-} me^v* stock in which the CD5 null allele was fixed to homozygosity and the mice were segregating for the *me^v* mutation. Concurrently, 129P3/J wild type mice were bred with C57BL/6-+/+*me^v* mice in order to produce *me^v/me^v* and +/+ control mice on a matched segregating background that expressed normal CD5 levels. Mice were typed for the CD5 null allele by PCR, using primers for the inserted neomycin resistance gene (*neo13*: 5'-

CTTGGGTGGAGAGGCTATTC-3', *neo14*: 5'-AGGTGAGATGACAGGAGATC-3').

Determination of the genotype at the *Hcph* locus (*me^v/me^v*, *+/me^v* or *+/+*) was also conducted by PCR, using primers flanking the viable motheaten mutation (*me^v*-F: 5'-CGTGTTCATCGTCATGACT-3', *me^v*-R: 5'-AGGAAGTTGGGGCTTTGCCGT-3'). Before electrophoresis, the amplification products were digested with *Rsa*I to distinguish among *+/+*, *+/me^v* and *me^v/me^v* mice (298). Mice were housed in conventional pathogen-free animal facilities (299).

Histopathology

Groups of mice from 4 – 18 wks of age were euthanized by CO₂ asphyxiation. Tissues were fixed in Fekete's acid alcohol formalin, embedded in paraffin, and sectioned at 6 µm. Slides were stained with Mayer's hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) for histological examination. Immunohistochemistry was carried out on lung and kidney sections using biotinylated rabbit anti-mouse Ig (DAKO, Carpinteria, CA) for identification of immune complexes. Non-specific staining was blocked on deparaffinized sections with peroxidase blocking reagent (DAKO). Tissues were then incubated in primary antibody for 1 h at room temperature in a humidified chamber. After three 5 min washes in PBS, tissues were incubated with avidin/horseradish-peroxidase conjugate for 45 min, followed by an additional trio of 5 min washes in PBS. Tissues were then incubated in diaminobenzidine (DAB) for 5 min at room temperature and washed in tap water for 5 to 15 min. Tissues were counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

Flow cytometric analysis

Monoclonal antibodies, conjugated with phycoerythrin, FITC or biotin, were used for characterization of spleen cells from 9 – 12 wk-old and 15 – 18 wk-old mice. Streptavidin-Red 6-70 was used as a secondary fluorophore for biotinylated antibodies. The following antibodies were obtained from PharMingen, Inc. (San Diego, CA): anti-CD4, clone RM4-5; anti-pan granulocyte (Gr-1), clone RB6-8C5 (300); anti-Mac-1, clone M1/70 (301); anti-CD23, clone B3B4 (302, 303); anti-CD43, clone S7 (304-306); and anti-CD19, clone 1D3 (307-309). Additional antibodies, as follows, were purified from hybridoma cell lines as ascites; anti-CD3, clone 145-2C11 (310); anti-CD8, clone 53-6.72 (311, 312); anti-IgM, clone R6-60.2; anti-B220, clone RA3-6B2 (313); anti-pan macrophage, clone F4/80; anti-MHC class I, clone M1/42 (314); anti-MHC class II, clone M5/114 (315); anti-pan erythrocyte, clone Ter119 (316); and anti-CD5, clone 53-7.3 (311, 312). Single cell suspensions were prepared from individual spleens by extrusion of cells through Nytex 110 mesh bags (TETKO Inc., Elmsford, NY) into cold HBSS containing 5% FBS and 0.1% sodium azide. Erythrocytes were lysed in buffered ammonium chloride, and leukocyte populations were phenotyped as previously described (297) using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). B-1 B-cells were identified by labeling cells with antibodies against IgM, CD19, and CD43. Cells that were IgM^{high}, CD19⁺, CD43⁺ were considered B-1 B-cells (317, 318).

Ig levels

Levels of IgM, IgG1, and IgG3 in serum from 9 – 12 wk-old individual mice were assayed by ELISA as previously described (297). Plates were coated with goat anti-mouse isotype specific antibody (Southern Biotechnology Associates, Birmingham, AL). Alkaline phosphatase labeled goat anti-mouse κ -chain (Southern Biotech) was used as the detection antibody. Isotype specific standards (PharMingen) were run with each assay. Plates were developed with p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) and read with the EL 312e Bio-Kinetics Reader (Bio-Tek Instruments, Winooski, VT). Ig levels were determined from the standard curves.

Autoantibody determinations by ELISA

Circulating autoantibodies against histone proteins were determined using a modification of a protocol described by Amoura *et al.* (319). In brief, individual wells of 96 well microtiter plates were first coated with 100 μ l of 2 μ g/ μ l calf thymus histone protein (Type IIS, Sigma) in PBS for 1 h at 37°C. The wells were then washed in an automated microplate washer with PBS containing 0.25% Tween 20 (PBS/T20), and then blocked with 200 μ l PBS containing 1% BSA for 1 h at 37°C. The wells were washed, and 100 μ l of each serum dilution was added to the appropriate wells. After incubation for one additional hour at 37°C, the wells were washed three times, and then 100 μ l of a previously titrated goat anti-mouse IgG (H+L)-alkaline phosphatase conjugate was added to each well. Following incubation at 37°C for 1 h, the plates were washed, and then 100 μ l substrate (p-nitrophenyl phosphate, Sigma) dissolved at 1 mg/ml in diethanolamine substrate buffer was added to each well. The rate of substrate conversion to colored

product at 37°C was monitored at 405 nm and recorded as mOD/min. Autoantibodies against double stranded DNA were detected according to a modification of a protocol described by Zouali *et al.* (320). In brief, microtiter wells were coated with calf thymus DNA at 10 µg/ml in TBS (10 mM Tris base, 150 mM NaCl, pH 7.4) for 2 h at room temperature. Following this incubation, the plates were washed with PBS/T20 in an automated microplate washer, and then processed as described above.

Hematology

Blood was collected from the retro-orbital sinus using heparinized capillary tubes. Leukocytes and erythrocytes were counted using a model ZBI Coulter counter (Hialeah, FL). Mean corpuscular volumes (MCVs) were calculated from the packed red cell volumes and total RBC counts as previously described (250). Blood smears were stained with Wright-Giemsa (Sigma) for examination of cells for morphological abnormalities. Reticulocytes were counted using the ADVIA 120 Hematology system (Bayer Corp, Tarrytown, NY). Confirmation of reticulocyte percentages was conducted manually following staining with New Methylene Blue (NMB) stain (Mallinckrodt Baker Inc., Phillipsburg, NJ). Equal amounts of blood and stain were incubated together in a capillary tube for 15 min and smears were prepared. The smears were then air-dried and coverslipped with Permount (Fisher).

Spleen cell lysates and immunoblotting

Spleens were dissected from mutant and control mice at 5 – 8 wks of age and at 12 – 15 wks of age. Single cell suspensions were prepared after lysis of red blood cells

following established procedures (321). For immunoblotting, the spleen cells were lysed at 4°C for 30 min in cold lysis buffer (20 mM Tris, pH 7.4; 1 mM EDTA; 10% glycerol; 1% Triton X-100; 100 mM NaCl; 1 µg of leupeptin per ml; 1 µg of aprotinin per ml; 1 mM benzamide and 5 mM of iodoacetic acid). Cell lysates were clarified by centrifuging for 10 min at 10,000g at 4° C. Protein levels were measured using the DC Protein Assay (Bio-Rad, Hercules, CA). 20 µg of each sample was resolved in a 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were probed, as described previously (322), with monoclonal antibody against phosphotyrosine (4G10, Upstate Biotechnology Inc., Lake Placid, NY). Specific antibody signals were detected using an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden).

Bone marrow macrophage cultures

Bone marrow plugs from femurs and tibias were harvested by extrusion with cold HBSS. Marrow plugs were disrupted by passage through a 25g needle. The resulting single cell suspensions were washed 2x in sterile HBSS (Sigma), counted with a model ZBI Coulter counter and resuspended in complete media (RPMI-1640 (Sigma) containing 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin). A total volume of 5 ml medium containing 10⁶ cells/ml was added to 25 cm² tissue culture flasks with either 500 or 1000 units/ml of recombinant human (rHu) CSF-1 (Cetus) or recombinant murine (rMu) GM-CSF (R&D Systems, Minneapolis, MN). Cultures were incubated at 37°C in 5% CO₂ for 24 h to allow adherent cells to attach to the flask. The non-adherent cells were transferred to duplicate flasks and cultured for 7 days with

periodic changes of media, maintaining the appropriate growth factor concentrations. To recover adherent cells from flasks, medium containing any non-adherent cells was removed and transferred to 50 ml tubes. The remaining adherent cells were washed 2x with 10 ml warm HBSS, then incubated for 10 min with 10 ml 2mM EDTA/PBS at 37°C. Cultures were vigorously shaken to detach all adherent cells. The adherent cells were then combined with the non-adherent cells in 50 ml tubes, washed 2x in HBSS and used for FACS analysis as previously described. Preliminary FACS analysis of adherent and non-adherent cells separately showed that they had similar staining profiles. In subsequent FACS analyses, adherent and non-adherent were combined to increase the numbers of cells available for analysis.

Statistics

All measures of variance are presented as SEM. Student's *t* tests were performed to determine significance of difference of means. Significance was assumed for *p* values < 0.05.

Results

Longevity

Thirty five B6;129-CD5^{-/-} *me^v/me^v* mice and an equal number of B6;129-*me^v/me^v* mice were monitored daily from three weeks of age (Figure 3.2). There was no significant effect of gender on survival of these mice. The mean lifespan of B6;129-CD5^{-/-} *me^v/me^v* mice (162 +/- 10 d) was significantly increased in comparison to B6;129-*me^v/me^v* mice, which had a mean lifespan of 89 +/- 8 d (*p*<0.0001). Our previous studies

revealed that C57BL6- me^v/me^v mice had a mean lifespan of 61 ± 2.4 d (250), indicating an effect of strain background on lifespan of these mice. All subsequent data compares me^v/me^v mice, CD5 $^{-/-}$ me^v/me^v mice and wild type $+/+$ controls on the B6;129 segregating background. Although lifespan data has not been gathered on wild type B6;129 mice, the average lifespan of C57BL/6J mice is 800 days for males and 750 days for females (323).

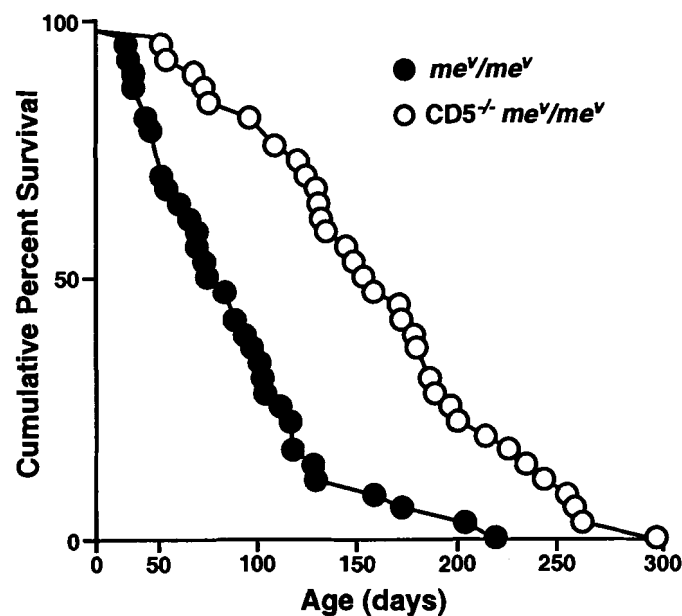


Figure 3.2. Cumulative percent survival of B6;129- me^v/me^v and B6;129-CD5 $^{-/-}$ me^v/me^v mice as a function of age. Thirty-five male or female mice from each genotype were monitored from 3 wks of age.

Immunopathology

In order to determine the effect of the CD5 null allele on the onset and progression of the characteristic pathologic lesions of *me^v/me^v* mice, total necropsies were performed on mutant (CD5^{-/-} *me^v/me^v* and *me^v/me^v*) and control (CD5^{-/-} +/? and +/?) mice. All tissues were examined histologically with a pathologist (J.P.S.) (324). Several striking differences were noted in B6;129-CD5^{-/-} *me^v/me^v* mice when compared to B6;129-*me^v/me^v* mice.

Development of pulmonary lesions was significantly delayed in CD5^{-/-} *me^v/me^v* mice in comparison to *me^v/me^v* mice. At 9 wks of age, this difference was evident grossly at the time of necropsy. Lungs of *me^v/me^v* mice were mottled tan in color and firm, while lungs of CD5^{-/-} *me^v/me^v* mice resembled the lungs of wild type mice and were compliant and evenly light pink in color. Histologically, the *me^v/me^v* mice developed earlier acidophilic macrophage pneumonia (325, 326), which progressed in severity more rapidly than in CD5^{-/-} *me^v/me^v*. Moderate disease was evident by 4 wks of age in *me^v/me^v* mice and pneumonia was severe by 9 wks. Pulmonary lesions were minimal or absent in CD5^{-/-} *me^v/me^v* mice at 4 wks of age, and by 9 wks of age, only mild peribronchiolitis or pneumonitis was observed (Figure 3.3). By 21 wks of age, most CD5^{-/-} *me^v/me^v* mice developed mild to moderate acidophilic macrophage pneumonia, while one CD5^{-/-} *me^v/me^v* mouse necropsied at 43 wks of age had only mild pulmonary disease (data not shown).

Spleens in CD5^{-/-} *me^v/me^v* mice were markedly enlarged. Spleen weight expressed as a ratio of spleen to body weight (S:B^{wt}) was increased 42% in CD5^{-/-} *me^v/me^v* mice in comparison to *me^v/me^v* mice (p<0.05) at 9 wks of age. The S:B^{wt} of *me^v/me^v* mice at 9

wks of age was increased almost 7 fold over a control group of +/+ and +/- mice combined (data not shown). The S:B^{wt} of both CD5^{-/-} *me^v/me^v* mice and *me^v/me^v* mice increased with age from 5 – 18 wks, while in +/- and CD5^{-/-} +/- mice, this ratio did not change considerably with age. The spleens in CD5^{-/-} *me^v/me^v* mice at 9 wks of age had poorly defined follicles and contained numerous multinucleated giant cells with abundant cytoplasm. While the splenic follicles of *me^v/me^v* mice were poorly delineated, the spleens lacked the extensive granulomatous lesions, consisting of multinucleated giant cells, found in CD5^{-/-} *me^v/me^v* mice (Figure 3.4). With increasing age, multinucleated giant cells in CD5^{-/-} *me^v/me^v* mouse spleens became even more abundant, while the relative number of these cells in spleens of *me^v/me^v* mice increased only slightly.

The livers in CD5^{-/-} *me^v/me^v* mice showed marked extramedullary myelo- and erythropoiesis at 9 wks of age. In contrast, the livers in *me^v/me^v* mice exhibited only mild extramedullary hematopoiesis (EMH), mostly myeloid in nature (Figure 3.5). By 16 wks of age, EMH in livers of CD5^{-/-} *me^v/me^v* mice had increased considerably, while EMH in livers of *me^v/me^v* mice remained mild to moderate (data not shown).

CD5^{-/-} *me^v/me^v* mice did not develop hyperplasia of the non-glandular stomach, a feature commonly seen in *me^v/me^v* mice at 9 wks of age (Figure 3.6 A). Inflammation of the glandular stomach was observed in both CD5^{-/-} *me^v/me^v* mice and *me^v/me^v* mice. Renal glomeruli of CD5^{-/-} *me^v/me^v* mice were enlarged and increased in cellularity at 9 wks of age, suggesting a mild membranoproliferative glomerulonephritis. The *me^v/me^v* mice exhibited glomerulonephritis, but the condition was less severe (Figure 3.6 B). Immune complexes were detected in the glomeruli of both CD5^{-/-} *me^v/me^v* and *me^v/me^v* kidneys by PAS staining and by immunohistochemistry using anti-mouse Ig (data not shown).

Blood urea nitrogen (BUN) levels were not significantly elevated in either $CD5^{-/-} me^v/me^v$ or me^v/me^v mice ranging from 6 to 12 wks of age (data not shown).

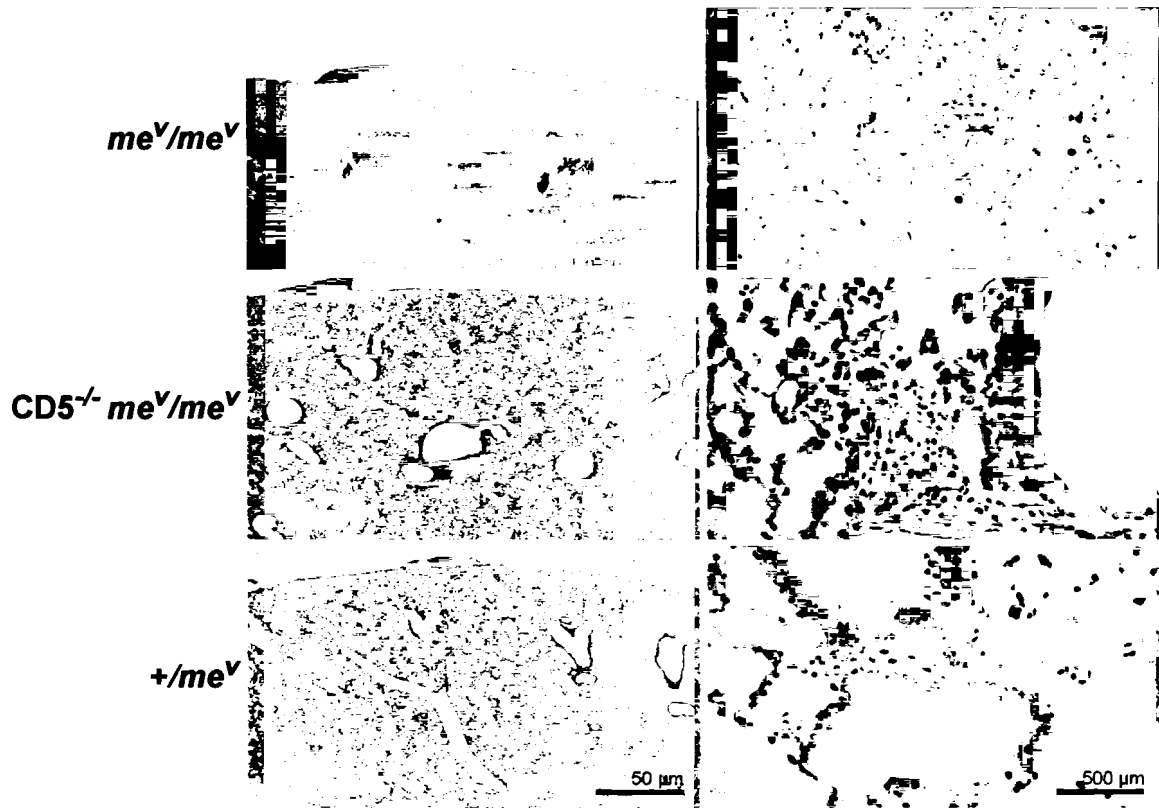


Figure 3.3. Photomicrographs of lung of $CD5^{-/-} me^v/me^v$ mice. Lung shows severe eosinophilic macrophage pneumonia in me^v/me^v mice, in contrast to minor peribronchiolar aggregation of lymphocytes in $CD5^{-/-} me^v/me^v$ mice and normal appearance in $+/me^v$ control mice. Tissue sections are from 62-64 day old B6;129 mice. All tissues were fixed in Fekete's acid alcohol formalin and stained with hematoxylin and eosin.

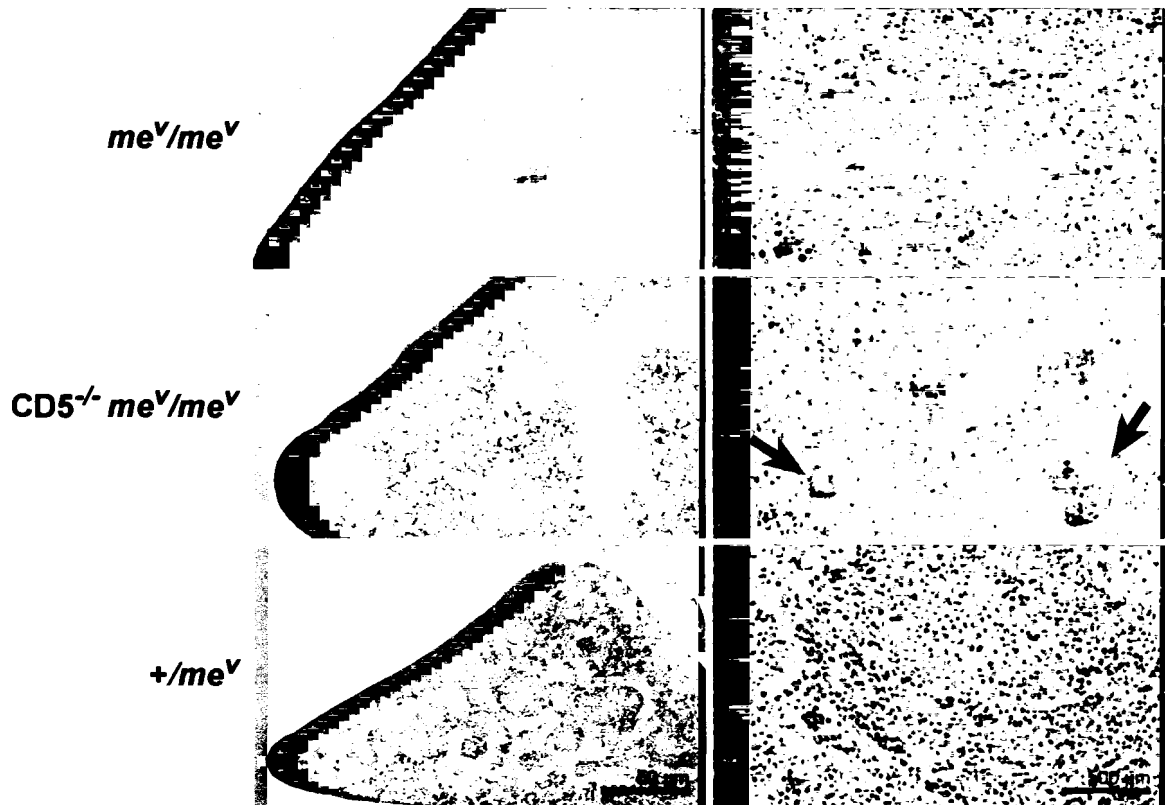


Figure 3.4. Photomicrographs of spleen of $CD5^{-/-} me^v/me^v$ mice. Spleens of me^v/me^v and $CD5^{-/-} me^v/me^v$ mice have poorly defined lymphoid follicles that are markedly depleted of lymphoid cells. Spleens of $CD5^{-/-} me^v/me^v$ mice contain many multinucleated giant cells (arrows) that are not present in me^v/me^v spleens. In $+/me^v$ mice, spleens reveal well-developed follicles. Tissue sections are from 62-64 day old B6;129 mice. All tissues were fixed in Fekete's acid alcohol formalin and stained with hematoxylin and eosin.

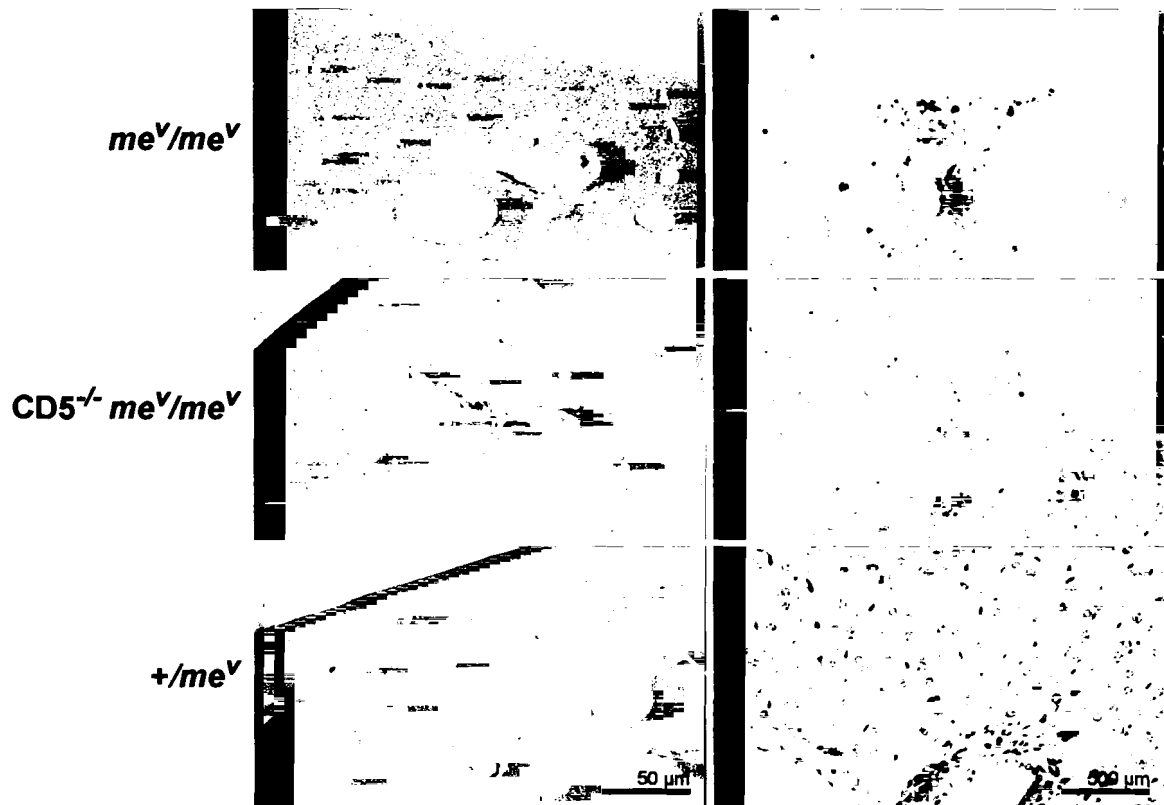


Figure 3.5. Photomicrographs of liver of $CD5^{-/-} me^v/me^v$ mice. Livers of me^v/me^v mice exhibit mild extramedullary hematopoiesis, mostly myeloid in nature. In contrast, high levels of extramedullary myelo- and erythropoiesis are evident in livers of $CD5^{-/-} me^v/me^v$ mice. The $+/me^v$ livers appear normal. Tissue sections are from 62-64 day old B6;129 mice. All tissues were fixed in Fekete's acid alcohol formalin and stained with hematoxylin and eosin.

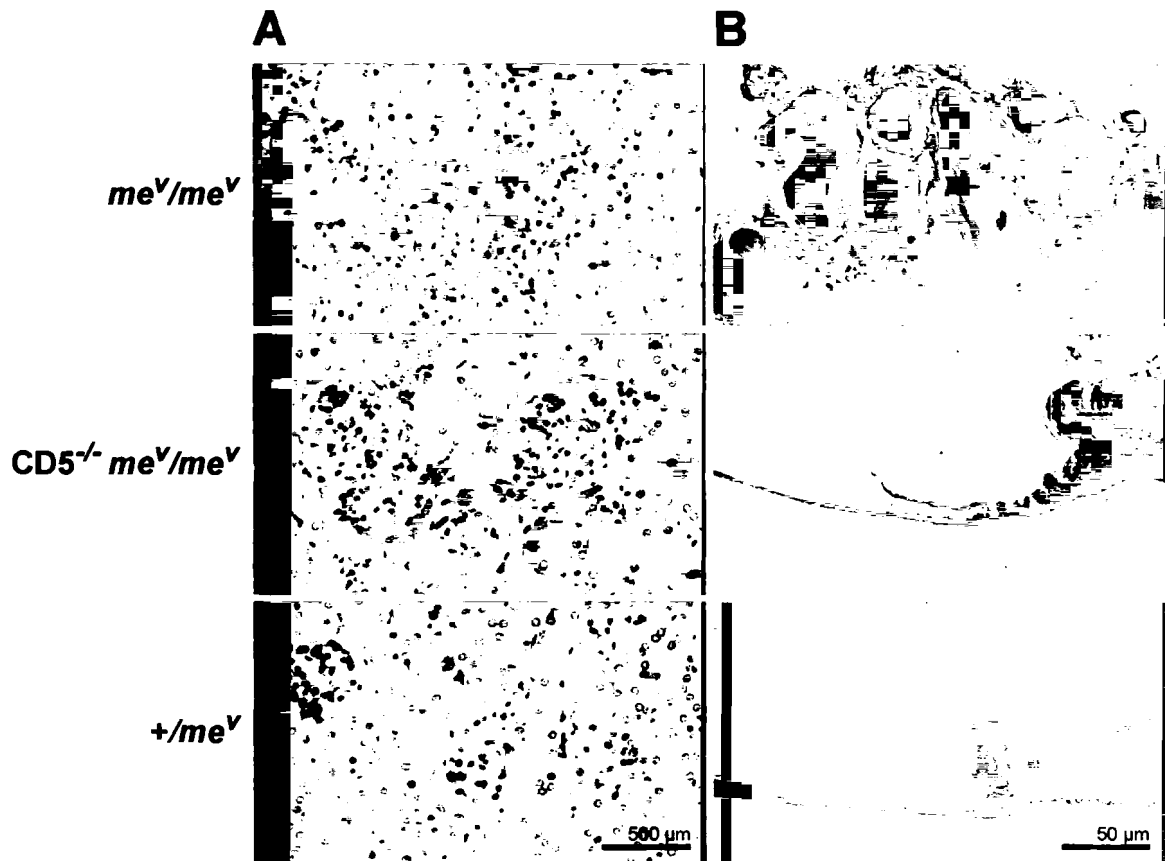


Figure 3.6. Photomicrographs of kidney and stomach of *CD5^{-/-} me^v/me^v* mice. A) Renal glomeruli of *CD5^{-/-} me^v/me^v* mice are enlarged and increased in cellularity, suggesting of a mild membranoproliferative glomerulonephritis. The *me^v/me^v* mice show less severe glomerulonephritis. B) Hyperplasia of the non-glandular stomach commonly seen in *me^v/me^v* mice is not present in *CD5^{-/-} me^v/me^v* mice. Tissue sections are from 62-64 day old B6;129 mice. All tissues were fixed in Fekete's acid alcohol formalin and stained with hematoxylin and eosin.

Flow cytometry analyses of cell populations

The observed splenomegaly in $CD5^{-/-} me^v/me^v$ and me^v/me^v mice was accompanied by a significant increase in total numbers of nucleated cells in the spleen in comparison to $CD5^{-/-} +/+$ and $+/+$ mice, respectively ($p < 0.05$). Average numbers of nucleated splenocytes in $CD5^{-/-} me^v/me^v$ were increased in comparison to me^v/me^v mice, but the data were not statistically significant. An elevation in the number of splenic myeloid cells contributes to the splenomegaly observed in me^v/me^v mice (250). While an increase in ratio of spleen weight to body weight (S:B^w) was observed in $CD5^{-/-} me^v/me^v$ mice in comparison to me^v/me^v mice, the reduction in severity of acidophilic macrophage pneumonia observed in $CD5^{-/-} me^v/me^v$ mice suggested a general decrease in myeloid cells numbers. To determine the proportions of cell populations present in the spleens of $CD5^{-/-} me^v/me^v$ mice, flow cytometric analysis was performed.

Percentages of splenic Mac-1⁺ Gr-1⁺ monomyeloid cells were significantly reduced in $CD5^{-/-} me^v/me^v$ mice compared to me^v/me^v mice, as were percentages of IgM⁺ B220⁺ B-cells and CD3⁺ CD4⁺ T-cells (Table 3.1). However, $CD5^{-/-} me^v/me^v$ mice had a significant increase in percentages of splenic Ter119⁺ erythrocyte precursor cells compared to me^v/me^v mice, suggesting that the augmented splenomegaly in $CD5^{-/-} me^v/me^v$ mice is a result of an increase in erythropoiesis.

Table 3.1. Flow cytometry analyses of spleen cells from 9 – 12 wk-old B6;129-CD5^{-/-} *me^v/me^v* mice.

Cell Population	B6;129 <i>me^v/me^v</i>	B6;129 CD5 ^{-/-} <i>me^v/me^v</i>	B6;129 +/?	B6;129 CD5 ^{-/-} +/?
CD3 ⁺ CD4 ⁺	8.9 ± 1.0	3.2 ± 1.5*	16.1 ± 1.8	17.5 ± 3.5
CD3 ⁺ CD8 ⁺	2.6 ± 0.8	1.1 ± 0.5	8.2 ± 1.0	9.9 ± 1.1
Mac-1 ⁺ Gr-1 ⁺	40.2 ± 3.0	14.2 ± 2.5*	6.1 ± 1.2	5.1 ± 1.7
Mac-1 ⁺ Gr-1 ⁻	21.2 ± 3.4	29.9 ± 3.9	12.5 ± 7.9	15.7 ± 5.4
Mac-1 ⁻ Gr-1 ⁺	4.7 ± 1.5	1.1 ± 0.4*	4.3 ± 0.6	6.3 ± 2.0
IgM ⁺ B220 ⁺	19.5 ± 2.2	7.1 ± 2.7*	56.1 ± 5.2	52.4 ± 5.2
B-1 cells [†] (as % of total B-cells)	79.6 ± 5.0	83.6 ± 5.7	5.4 ± 0.6	6.6 ± 0.6
Ter119 ⁺	12.6 ± 2.9	56.2 ± 7.8*	2.2 ± 1.1	2.2 ± 0.8
Data are expressed as a mean percent ± SEM {n = 6 for B6;129-CD5 ^{-/-} <i>me^v/me^v</i> ; n = 5 for B6;129- <i>me^v/me^v</i> ; n = 4 for B6;129-CD5 ^{+/+} +/? and B6;129-+/? } { [†] B-1 cells were identified as IgM ^{high} , CD19 ⁺ , CD43 ⁺ } { * indicates significant (p < 0.05) difference in spleen cell populations between CD5 ^{-/-} <i>me^v/me^v</i> and <i>me^v/me^v</i> mice }				
Mean # of nucleated spleen cells x 10 ⁸	3.4 ± 0.6	4.4 ± 0.7	1.7 ± 0.3	1.1 ± 0.1

To determine whether CD5 is necessary for development of B-1 cells, percentages of these cells were determined using antibodies against IgM, CD43, and CD19.

Percentages of IgM⁺ B220⁺ B-cells that had a B-1 phenotype did not vary significantly between CD5^{-/-} *me^v/me^v* mice and *me^v/me^v* mice (Figure 3.7), indicating that CD5 expression is not required for development of the B-1 cell population.

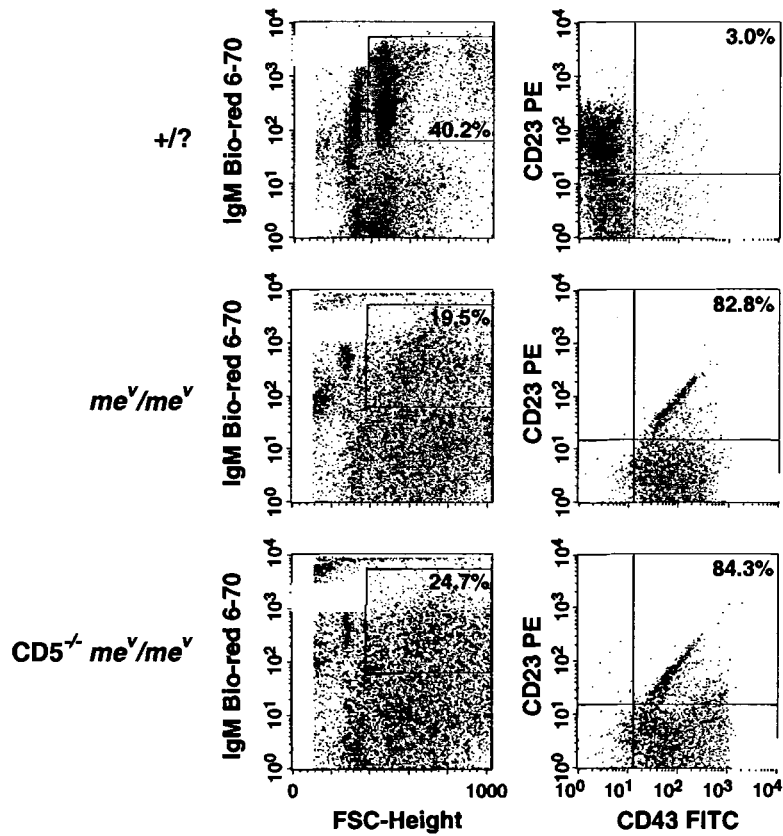


Figure 3.7. Flow cytometry analysis of B-1 B cells in spleens of $CD5^{-/-} me^v/me^v$, me^v/me^v , and $+/?$ mice. Panels on the left show percentage of IgM^{hi} cells. Panels on the right show percentage of IgM^{hi} cells that are $CD43^{+}/CD23^{-}$, an expression pattern characteristic of B-1 B cells.

Total nucleated cell numbers in the spleen did not change significantly with age in $CD5^{-/-} me^v/me^v$, me^v/me^v , $CD5^{-/-} +/-$ or $+/?$ mice. The only significant change in percentages of spleen cell populations in $CD5^{-/-} me^v/me^v$ with age was a decrease in the

percentage of Mac-1⁺ Gr-1⁺ cells ($p < 0.05$) in 15 – 18 wk-old mice in comparison to 9 – 12 wk-old mice (data not shown). In contrast, several significant differences were seen when comparing spleens from 9 – 12 wk-old *me^v/me^v* mice to spleens of 15 – 18 wk-old *me^v/me^v* mice. Percentages of CD3⁺ CD4⁺ T-cells, Mac-1⁺ Gr-1⁺ cells and IgM⁺ B220⁺ B-cells were lower in 15 – 18 wk-old *me^v/me^v* mice when compared to 9 – 12 wk-old *me^v/me^v* mice ($p < 0.05$), while the percentage of Ter119⁺ cells was increased ($p < 0.01$) (data not shown).

Peripheral blood analysis

Analysis of reticulocyte levels in peripheral blood using the Advia 120 Hematology Analyzer showed a significant increase in reticulocyte percentages in CD5^{-/-} *me^v/me^v* mice when compared to *me^v/me^v* mice ($p < 0.05$) (Table 3.2). Reticulocyte percentages of both *me^v/me^v* and CD5^{-/-} *me^v/me^v* mice were significantly higher than in wild type controls ($p < 0.05$). Reticulocytes were also examined in smears of blood stained with NMB, as described (Figure 3.8). This data reflects the results found using the Advia 120 Hematology Analyzer.

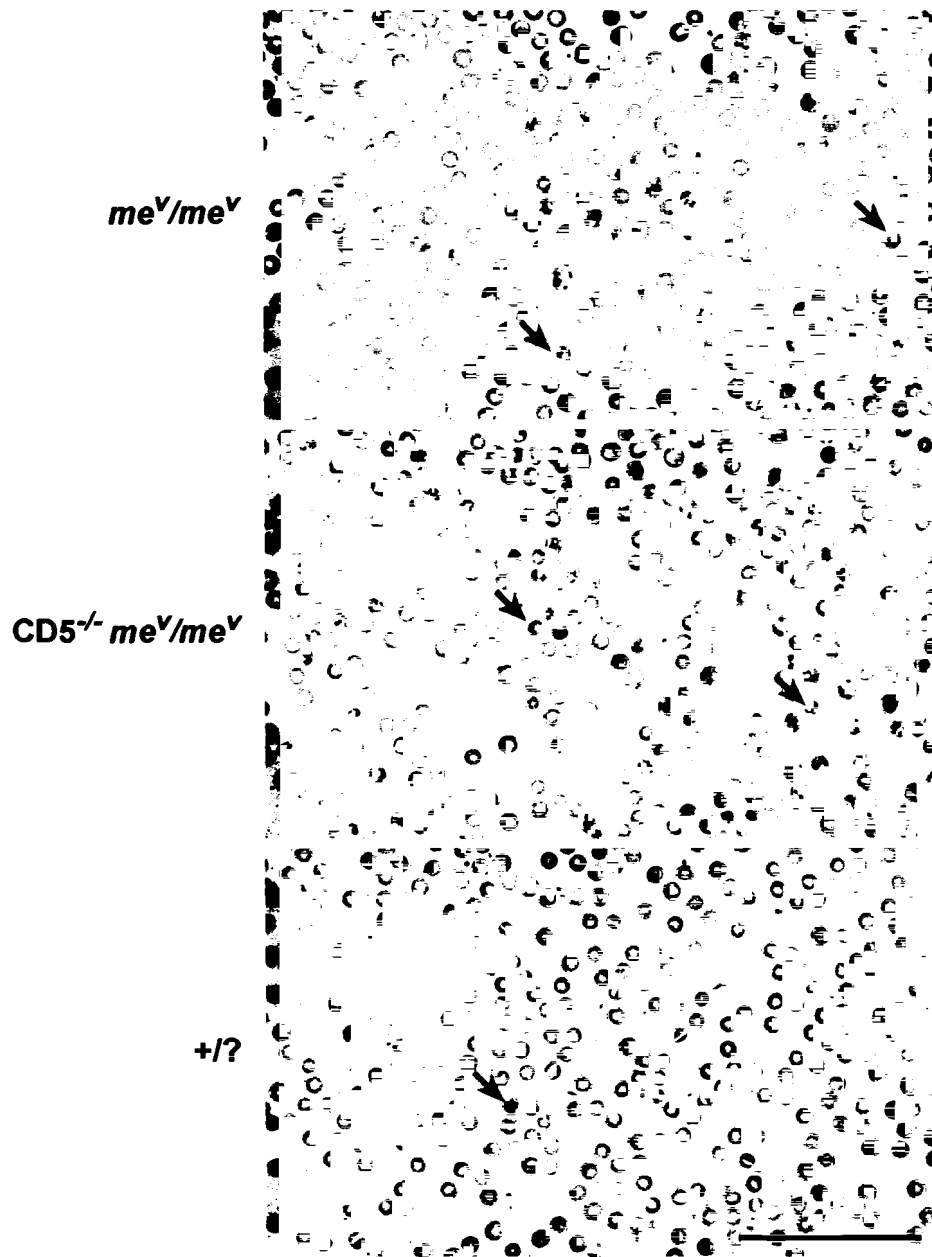


Figure 3.8. New methylene blue (NMB) staining for reticulocytes. Reticulocytes contain RNA, which precipitates in NMB and is stained blue. Reticulocytes have discernible blue granules (arrows). A manual reticulocyte count may be done by counting red cells in a smear of new methylene blue stained blood and calculating the percentage of total red cells that contain blue granules (reticulocytes).

In contrast to reticulocyte numbers, peripheral blood red cell numbers were significantly reduced in both $CD5^{-/-} me^v/me^v$ and me^v/me^v mice in comparison to wild type (+/+ and +/-) controls at 9 – 12 wks of age ($p < 0.05$). Although there was a significant increase in numbers of Ter-119⁺ splenic nucleated erythroid cells in $CD5^{-/-} me^v/me^v$ mice in comparison to me^v/me^v mice (Table 3.1), there was not a corresponding increase in peripheral erythrocyte counts in $CD5^{-/-} me^v/me^v$ mice (Table 3.2). Moreover, there were no significant differences in peripheral erythrocyte or leukocyte counts between $CD5^{-/-} me^v/me^v$ and me^v/me^v mice, although the average erythrocyte and leukocyte numbers as well as hematocrit percentages were slightly lower in $CD5^{-/-} me^v/me^v$ mice in comparison to me^v/me^v mice. Hematocrit percentages of me^v/me^v mice were lower on average than those of wild type (+/+ and +/-) mice, but the difference lacked statistical significance by a small margin ($p = 0.055$). Although the decrease in hematocrit percentages of $CD5^{-/-} me^v/me^v$ versus me^v/me^v mice was also not statistically significant, $CD5^{-/-} me^v/me^v$ mice did have significantly lower hematocrits than +/+ or +/- mice ($p < 0.0001$). Erythrocyte mean cell volumes (MCV) did not differ significantly in any of the genotypes studied (data not shown). Peripheral blood leukocyte numbers were comparably increased in both $CD5^{-/-} me^v/me^v$ mice and me^v/me^v mice compared to wild type controls. The majority of these cells were monocytes and granulocytes (data not shown). There was no significant difference in either leukocyte or erythrocyte numbers in $CD5^{-/-} +/-$ mice versus wild type +/- mice.

Table 3.2. Peripheral blood analysis of 9 – 12 wk-old CD5^{-/-} *me^v/me^v* mice.

Cell Population	B6;129 <i>me^v/me^v</i>	B6;129 CD5 ^{-/-} <i>me^v/me^v</i>	B6;129 +/?	B6;129 CD5 ^{-/-} +/?
Erythrocytes (x 10 ⁹ cells/ml)	7.0 ± 0.6	6.4 ± 0.4	9.3 ± 0.8	9.7 ± 0.7
Leukocytes (x 10 ⁶ cells/ml)	16.6 ± 3.0	14.2 ± 2.0	7.5 ± 0.8	9.1 ± 1.4
Hematocrit (%)	44.0 ± 2.8	39.4 ± 1.4	50.3 ± 1.5	51.5 ± 0.7
Reticulocytes (as % of total red blood cells)	17.3 ± 4.5	30.3 ± 5.2*	2.9 ± 0.1	2.8 ± 0.2

Data are expressed as stated ± SEM (n >3 for reticulocyte percentages; n > 6 for all other assays.) { * indicates significant (p < 0.05) difference in peripheral blood cell populations between CD5^{-/-} *me^v/me^v* and *me^v/me^v* mice }

Serum Ig levels

B-cells in *me^v/me^v* mice are hyperresponsive to stimulation through the B-cell receptor (BCR) (327). This hyperreactivity to BCR mediated signals in the absence of SHP-1 helps explain the otherwise counter-intuitive observation that serum levels of IgM and IgG in *me^v/me^v* mice are significantly higher than in wild type mice, while their mature B-cell numbers are reduced. Since CD5^{-/-} *me^v/me^v* mice have significantly fewer B-cells than *me^v/me^v* mice, we assayed serum Ig levels in CD5^{-/-} *me^v/me^v* and control mice (Figure 3.9).

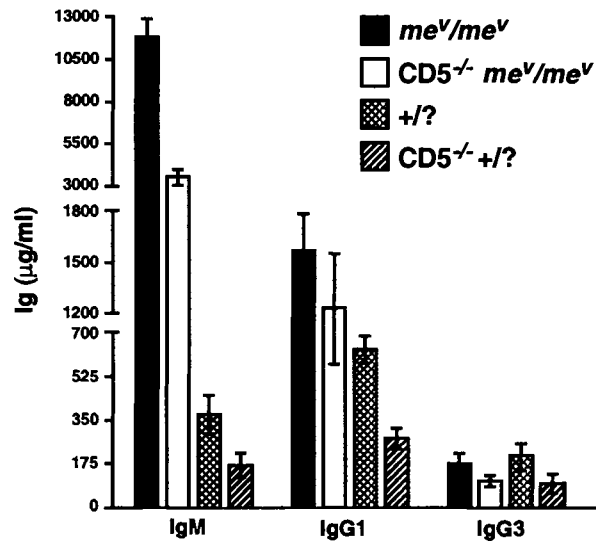


Figure 3.9. Serum immunoglobulin levels. Levels of serum IgM are significantly reduced in *CD5^{-/-} me^v/me^v* mice in comparison to *me^v/me^v* mice ($p < 0.05$). Absence of CD5 did not result in a significant reduction of IgG1 levels or IgG3 levels in *CD5^{-/-} me^v/me^v* mice in comparison to *me^v/me^v* mice. *CD5^{-/-} +/?* mice showed a consistent reduction in Ig levels in comparison to wild type (*+/?*) mice. However, the difference was only statistically significant when comparing IgG1 levels of *CD5^{-/-} +/?* mice versus *+/?* mice with intact CD5.

IgM levels were increased over 30 fold in *me^v/me^v* mice when compared with wild type (*+/+* and *+/me^v*) mice. In *CD5^{-/-} me^v/me^v* mice, IgM levels were 70% lower than in *me^v/me^v* mice but still almost 10 fold higher than in wild type (*+/+* and *+/me^v*) mice. Although previous reports have indicated that serum IgG1 levels in *C57BL/6J-me^v/me^v* mice are not significantly elevated over normal while serum IgG3 levels are significantly elevated in comparison with littermate controls (297), we found the opposite in our

studies. IgG1 levels were 2.5 times higher in the serum of *me^v/me^v* mice than in *+/?* littermates. These levels were lower in *CD5^{-/-} me^v/me^v* mice than in *me^v/me^v* mice, but the change was not statistically significant. No significant variation was observed in IgG3 levels between mutant and control mice.

It is interesting to note that levels of IgG1 were significantly reduced in *CD5^{-/-} +/?* mice in comparison to *+/?* mice with a functional CD5 gene. Production of IgG1 is T-cell dependent and CD5 expression by T-cells may be required to induce class switching or secretion of IgG1. Levels of IgM and IgG3 were lower in *CD5^{-/-} +/?* compared with *+/?* mice, but the differences were not statistically significant.

Autoantibody assays

To assess the presence of autoantibodies in *CD5^{-/-} me^v/me^v* and *me^v/me^v* mice, serum levels of anti-double stranded DNA (anti-dsDNA) and anti-histone antibodies were assayed by ELISA (Figure 3.10). Levels of these autoantibodies were elevated in the serum of both *CD5^{-/-} me^v/me^v* and *me^v/me^v* mice, when compared to wild type (*+/+* and *+/me^v*) controls. In contrast to IgM levels, that were significantly reduced in *CD5^{-/-} me^v/me^v* mice when compared to *me^v/me^v* mice, levels of both anti-dsDNA and anti-histone antibodies did not vary significantly between *CD5^{-/-} me^v/me^v* and *me^v/me^v* mice. Levels of anti-histone and anti-dsDNA antibodies in *CD5^{-/-} +/?* mice showed no variation from *+/+* and *+/me^v* controls.

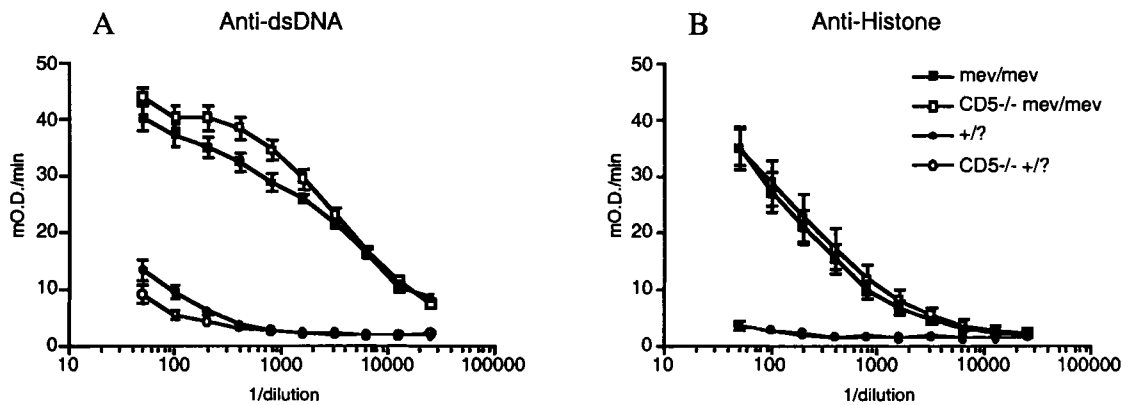


Figure 3.10. Autoantibody determination by ELISA. Data is expressed as an average rate of substrate conversion in mO.D./min \pm SEM (n=4).

Anti-phosphotyrosine western blotting

Variation in levels of tyrosine phosphorylation between two experimental samples can indicate a possible alteration in cell signaling. In order to explore whether the pathological changes observed in CD5^{-/-} *me^v/me^v* mice compared with *me^v/me^v* mice were accompanied by a difference in tyrosine phosphorylation, we examined total splenic protein by anti-phosphotyrosine western blotting (Figure 3.11). Spleen cell lysates were analyzed from mice at 12 – 15 wks of age as well as from mice at 5-8 wks (data from younger mice not shown). In *me^v/me^v* mice at 12 – 15 wks of age, there was an increase in tyrosine phosphorylation of proteins in the range of 28 – 45 kd. Highly phosphorylated proteins in this range were not seen in CD5^{-/-} *me^v/me^v* mice of any age, nor were they seen in younger *me^v/me^v* mice.

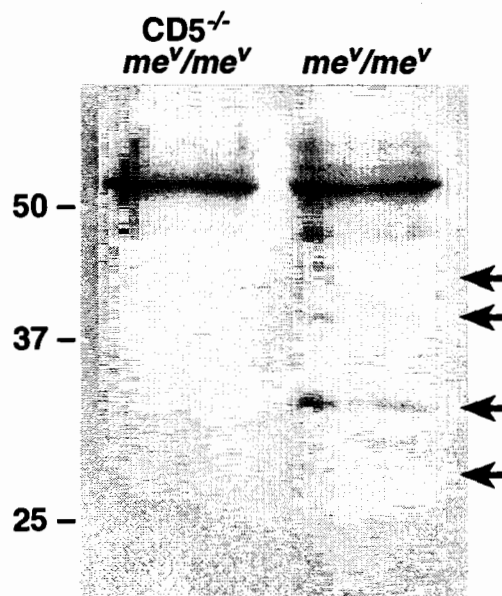


Figure 3.11. Characterization of tyrosine phosphorylation of proteins in spleen cell lysates. Spleen cell lysates from male C57BL/6J-CD5^{-/-} me^v/me^v and C57BL/6J-me^v/me^v mice at 4 months of age were analyzed by SDS-PAGE/immunoblotting with anti-phosphotyrosine antibody. The positions of protein size markers (kDa) are indicated on the left. Arrows indicate bands that exhibit enhanced tyrosine phosphorylation in C57BL/6J-me^v/me^v mice in comparison to C57BL/6J-CD5^{-/-} me^v/me^v mice.

Bone marrow macrophage cultures

Further support of the hypothesis that CD5 on macrophages plays a role in the development of the macrophage pneumonia seen in me^v/me^v mice required confirmation of the presence of CD5 directly on macrophages. Takahashi *et al.* (328) reported development of CD5 expression on macrophages in cell cultures grown with CSF-1 or

GM-CSF. To verify this, we established cultures of bone marrow cells from C57BL/6J-*me^v/me^v* mice as well as from C57BL/6J-+/? Controls and from C57BL/6J-CD5^{-/-} mice. Cultures were grown in the presence of either Rhu-CSF-1 or Rmu-GM-CSF. Adherent and non-adherent cells from cultures had similar cell surface marker expression profiles and were pooled for analysis. Flow cytometric analysis revealed that both C57BL/6J-+/? And C57BL/6J-*me^v/me^v* bone marrow contained cells that expressed CD5 after 7 days of culture with either 500 or 1000 units per ml of CSF-1 or GM-CSF (Figure 3.12). There was no difference in percentages of CD5⁺ cells with various amounts of cytokine, so results were pooled. Table 3.3 shows the mean results of three separate experiments. Following stimulation with GM-CSF, >90% of bone marrow cells that expressed CD5 were found to co-express Mac-1. Bone marrow from CD5^{-/-} mice grown under similar conditions, as expected, did not express CD5. Under all conditions, Gr-1 was expressed on <1.3% of CD5⁺ cells, and <0.9% of CD5⁺ cells expressed CD19 or CD3.

Table 3.3. Flow cytometry analysis of bone marrow cultures.

Cytokine	CSF-1		GM-CSF	
Genotype	+/?	<i>me^v/me^v</i>	+/?	<i>me^v/me^v</i>
% of total cells that are CD5 ⁺	6.1 ± 1.1	5.6 ± 0.9	4.0 ± 2.7	9.7 ± 2.7
% of CD5 ⁺ cells that are Mac-1 ⁺	49.1 ± 8.1	51.2 ± 6.3	61.8 ± 10.0	92.7 ± 3.0*

Data are expressed as a mean percent ± SEM. (n > 5) (* indicates significant (p<0.01) difference in % of CD5⁺ cells expressing Mac-1 between *me^v/me^v* cultures grown with GM-CSF and +/? cultures grown with GM-CSF as well as +/? and *me^v/me^v* cultures grown with CSF-1.)

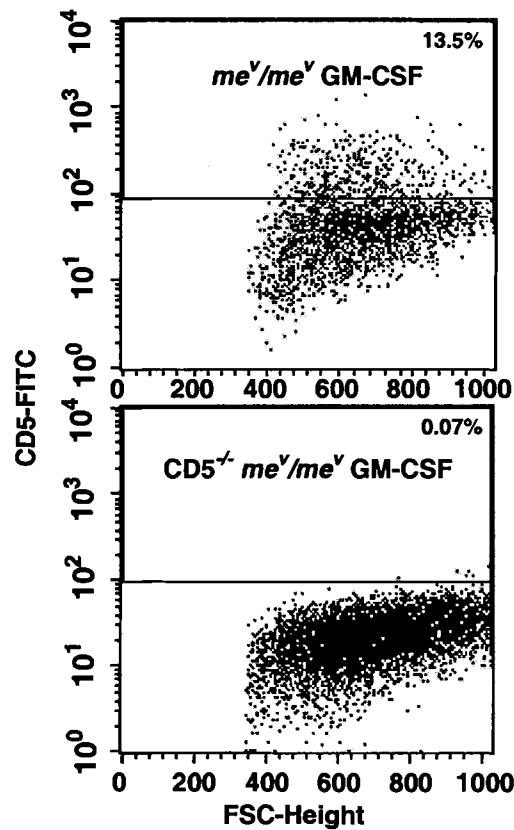


Figure 3.12. Expression of CD5 on bone marrow cells grown in culture for 7 days with 1000 units per ml GM-CSF. Top panel shows expression of CD5 on me^v/me^v cells, bottom panels shows lack of CD5 expression on $CD5^{-/-}$ cells. Dead cells were gated out using propidium iodide exclusion. Live cells were analyzed for CD5 expression. Numbers shown are percentages of $CD5^+$ cells.

Discussion

This study focused on the role of CD5 in immunopathologic changes in *me^y/me^y* mice. Absence of CD5 expression in *me^y/me^y* mice resulted in significantly increased lifespan and numerous phenotypic changes in comparison to *me^y/me^y* mice with an intact CD5 gene. Although a significant decrease in serum IgM levels was seen in CD5^{-/-} *me^y/me^y* mice, this was not associated with reductions in autoantibodies against dsDNA or histone proteins, two autoantibodies frequently elevated in *me^y/me^y* mice. The increased longevity of CD5^{-/-} *me^y/me^y* mice was, however, associated with a marked delay in development of acidophilic macrophage pneumonia in these mice when compared to *me^y/me^y* mice. The reduced levels of macrophage infiltration in the lungs of CD5^{-/-} *me^y/me^y* mice were associated with a significant decrease in myeloid cell numbers in the spleen. Although the reduced acidophilic macrophage pneumonia may be a consequence of altered interactions of B- or T-cells with myeloid cells due to the absence of CD5 on the surface of the lymphocytes, mature B- and T-cells are not necessary for the development of pulmonary inflammation in viable motheaten mice (329). Thus, the absence of CD5 function alone in either B- or T-cells would be unlikely to lead to such dramatic reduction of lung lesions as well as reduced myeloid cell populations in CD5^{-/-} *me^y/me^y* mice.

Recently, CD5 has been found to be constitutively expressed on a population of macrophages in *me^y/me^y* mice (281). CD5⁺ macrophages can be induced in the peritoneal cavity of wild type mice by treatment with high levels of GM-CSF. This cytokine is elevated in peritoneal fluid of *me^y/me^y* mice (328), and myeloid cells from *me^y/me^y* mice show enhanced signaling through the GM-CSF receptor (276). Our observation that

CD5^{-/-} *me^v/me^v* mice have reduced numbers of splenic myeloid cells and less severe myeloid cell mediated pulmonary lesions compared with *me^v/me^v* mice may indicate a role for CD5 in response of cells to myeloid growth factors such as GM-CSF. We confirmed the development of CD5⁺ macrophages in cultures of bone marrow from both *me^v/me^v* and wild type mice supplemented with either GM-CSF or CSF-1, providing support for the hypothesis that CD5 expression is involved with development or activation of macrophages through myeloid growth factor receptors. Although bone marrow cells from CD5^{-/-} mice grown in culture with GM-CSF or CSF-1 lacked CD5 expression, myeloid cell antigen (Mac-1 and Gr-1) expression profiles on these bone marrow cells were similar to cells from mice with an intact CD5 gene (data not shown). Additionally, no quantifiable or qualifiable differences were apparent in colony assays of bone marrow cells from CD5^{-/-} *me^v/me^v* mice or *me^v/me^v* mice grown in methylcellulose media with either CSF-1 or GM-CSF (data not shown). This data suggests that CD5 may play a role in later stages of myelopoiesis.

For many years, the presence of a common progenitor for B-cells and macrophages has been suggested (330-333). Reports of biphenotypic B/macrophage cells have appeared in current literature (334-336) and CD5⁺ B-cells have been shown to develop macrophage characteristics (337, 338), often in association with B-cell malignancy (339, 340) (reviewed in (333)). It has been shown that treatment of wild type mice with GM-CSF resulted not only in an increase in numbers of CD5⁺ macrophages, but also in an elevation in numbers of CD5⁺ hematopoietic progenitor cells and CD5⁺ B-cells (328), supporting the presence of a common precursor for CD5⁺ (B-1a) B-cells and CD5⁺ macrophages. Lack of CD5 on biphenotypic B/macrophage cells or on

macrophages believed to have evolved from B-1 B-cells may contribute to the reduced severity of lung lesions seen in CD5^{-/-} *me^v/me^v* mice in comparison to *me^v/me^v* mice. CD5 expression may play a role in lineage commitment, possibly through altering responses to growth factors as suggested above.

There are three reported ligands for CD5 that are expressed on B-cells. These ligands include CD72, CD5 ligand, and cell surface Ig. Interactions between CD5 and these ligands are thought to play a role in communication between T and B-cells. CD72 is expressed constitutively on B-cells (341, 342), while CD5 ligand (CD5L, gp40-80) is expressed on activated B lymphocytes in the spleen (343) and is constitutively expressed on peritoneal B-cells and B lymphoma cell lines (344). Pospisil *et al.* (345) suggested that CD5 can also interact with certain sequences in the V_H framework of cell surface Igs. Ligation of T-cell surface CD5 to gp40-80 on B-cells has been implicated in the stimulation of B-cell proliferation through the gp40-80 receptor (344).

Until recently, CD5 ligands were reported to be present only on B-cells. However, several studies have now provided evidence that CD5 ligands are present on a variety of cell types in addition to B-cells, indicating that the interaction of CD5 with its various ligands may play a more diverse role in cellular communication than is currently thought. Agostini *et al.* reported the presence of CD72 on alveolar macrophages isolated from human sarcoidosis patients (346). A recombinant soluble protein based on the human CD5 extracellular region has been reported to bind to a variety of cells of myeloid as well as lymphoid origins (347), indicating the presence of a novel, widespread CD5 ligand. Just as CD5⁺ B-cells also express CD72, it is possible that a CD5 ligand is expressed on certain monomyeloid cells and that it may play a role in the activation of

these cells through binding with CD5. Alternatively, interaction of CD5 with an unknown ligand could be involved in adhesion of monomyeloid cells to other cell types. The extracellular region of CD5 is closely homologous to that of CD6 (348), a scavenger receptor family member involved in cell-cell adhesion through its ligand, CD166 (ALCAM) (349, 350). Interactions of CD5 with as yet unidentified ligands may be responsible for accumulation of macrophages in the lungs of motheaten mice due to dysregulated adhesion rather than solely dysregulated proliferation of pulmonary macrophages.

Red cells and mature myeloid cells arise from a common multipotential precursor, termed CFU-GEMM based on the ability of these progenitor cells to form granulocyte, erythroid, monocyte, and megakaryocyte colonies in culture in response to the appropriate cytokines. CFU-GEMMs can differentiate into either erythroid burst forming units (BFU-Es) in the presence of erythropoietin (Epo), or into granulocyte/macrophage colony forming units (CFU-GMs) in the absence of Epo but in the presence of myeloid growth factors such as GM-CSF. Spleens of *me^y/me^y* mice have previously been reported to contain high numbers of CFU-GMs in comparison to wild type mice, while the frequency of CFU-GMs in the bone marrow is similar in *me^y/me^y* and wild type mice (282). SHP-1 is involved in the negative regulation of Epo mediated signals (351). Both bone marrow and spleens of *me^y/me^y* mice contain increased numbers of erythroid precursors (CFU-Es) in comparison to wild type controls. CFU-Es from *me^y/me^y* mice are hyperresponsive to Epo and a subpopulation of *me^y/me^y* CFU-Es has lost their dependence on exogenous Epo (282). The increase in red cell precursors with a corresponding decrease in relative numbers of granulocytes in spleens of *CD5^{-/-} me^y/me^y*

mice versus *me^y/me^y* mice suggests the possibility that CD5 plays a significant role in the myeloid differentiation pathway in the absence of SHP-1. Dysregulated Epo signaling resulting from the absence of SHP-1 accompanied by insufficient myeloid growth factor mediated signals in the absence of CD5 could potentially explain the apparent shift from myelo- to erythropoiesis observed in *CD5^{-/-} me^y/me^y* mice in comparison to *me^y/me^y* mice. The lack of CD5 may cause changes in lineage commitment in the myeloid/erythroid branch of cellular development as a result of a reduced response to myeloid growth factors.

Although CD5 has been reported to be a negative regulator of signaling through both the T-cell receptor (290) and the B-cell receptor (352), earlier studies indicated a costimulatory role for CD5 in TCR signal transduction (353-355). Recently, additional studies have corroborated the reports of CD5 providing positive signals in both B- and T-cells, through a distinct cascade of second messengers including acidic sphingomyelinase and protein kinase C- ζ (356). Our finding that proteins in the range of 28 – 45 kd showed increased phosphorylation on tyrosine residues in spleens of older *me^y/me^y* mice but not in *CD5^{-/-} me^y/me^y* mice of the same age may provide additional evidence of a significant role in signaling for CD5. In the absence of the negative regulatory effects of SHP-1, the signals conveyed through CD5 may result in the phosphorylation of one or more novel signaling molecules in the 28 – 45 kd range. Identification of these molecules could help further elucidate the relationship between CD5 and SHP-1 and facilitate the resolution of the pathways in which these molecules interact.

Clear examination of the role of CD5 in the development of viable motheaten pathological lesions is problematic on the B6;129 segregating background. A

polymorphic variation between 129 and C57BL/6J linked to the *CD20* (*Ms4a2*) gene, which maps near the *CD5* locus, has been shown to affect peritoneal B-1 cell populations (357). Although the phenotypic differences observed in B6;129-*CD5*^{-/-} *me*^y/*me*^y mice in comparison to B6;129-*me*^y/*me*^y mice do not appear to be related to reduced B-1 cell numbers alone, the effect of strain specific polymorphisms is an important consideration in analyzing any targeted mutation on a segregating background.

We have recently completed backcrossing of the *CD5* mutation on to the C57BL/6J background (to N10) and have developed a colony of inbred C57BL/6J-*CD5*^{-/-} *me*^y/*me*^y mice. Spleen cell lysates from C57BL/6J-*CD5*^{-/-} *me*^y/*me*^y and C57BL/6J-*me*^y/*me*^y mice were utilized in the α -phosphotyrosine western blots shown in Figure 3.11. This experiment mirrored what was seen in extensive western blotting studies using B6;129-*CD5*^{-/-} *me*^y/*me*^y mice and B6;129-*me*^y/*me*^y mice. We are currently in the process of expanding our colony of C57BL/6J-*CD5*^{-/-} *me*^y/*me*^y mice. With these mice, we will re-examine the immunopathologic changes observed in our previous studies using B6;129 mice, as well as continue our investigation into the signaling alterations suggested by our reported western blotting results. Confirmation of the role of *CD5* in immunopathologic changes in *me*^y/*me*^y mice using inbred C57BL/6J-*CD5*^{-/-} *me*^y/*me*^y mice will mitigate the complications of variability associated with background modifying genes and polymorphic diversity.

Chapter 4

GENERATION OF C57BL/6J MICE CARRYING A TRANSGENE FOR MACROPHAGE SPECIFIC EXPRESSION OF DOMINANT NEGATIVE SHP-1 (C453S)

Introduction

The protein tyrosine phosphatase SHP-1, encoded by the hematopoietic cell phosphatase (*Hcph*) gene in mice (256, 257), is a negative regulator of signaling through a number of hematopoietic growth factor receptors (258, 276, 280). Much of the knowledge of the function of SHP-1 has been gained through the study of motheaten and viable motheaten mice. As previously discussed, mice homozygous for either of these two recessive mutations exhibit severe immunodeficiency and autoimmunity, and die at an early age from inflammatory lesions, encompassing accumulations of macrophages and neutrophils in the lungs and elsewhere (250-253). These mice have many cellular abnormalities, including an increase in myelopoiesis (250, 281) and multiple defects in lymphocyte development and function (282-285).

One of the challenges in the study of SHP-1 using motheaten mice has been discerning the cell autonomous functional defects caused by the absence of SHP-1 from the secondary effects of macrophages and neutrophils resulting in dysregulation of other cell populations. Previous attempts in our lab to transgenically rescue SHP-1 expression in specific cell populations in motheaten mice have been constrained by findings that cells overexpressing SHP-1 in these transgenic animals have a selective disadvantage in proliferation compared with the dysregulated cell populations that lack functional SHP-1

protein. Conversely, transgenic mice expressing a dominant negative or catalytically inactive form of SHP-1 under the control of cell specific promoters have been used successfully to study the effects of the absence of SHP-1 activity *in vitro* on individual cell types (358, 359).

Both *me/me* and *me^y/me^y* mice exhibit a marked expansion in numbers of monomyeloid cells. The importance of the SHP-1 phosphatase in regulating myeloid signaling pathways is evidenced by the severe inflammatory condition characteristic of motheaten mice. In order to dissect the cell autonomous defects caused by the lack of SHP-1 in macrophages, as well as to observe the effects of dysregulated macrophage populations on lymphoid and other myeloid cell populations, we created a mouse transgenic for macrophage-specific expression of a dominant negative form of SHP-1.

SHP-1 mutant C453S cDNA has been used previously to produce a dominant negative protein in transgenic studies (358-360). The basis of this catalytically inactive dominant negative mutant was the identification of an essential cysteine residue at position 453 within the catalytic domain of SHP-1 (361). This essential cysteine, when mutated to a serine (Figure 4.1), results in a expression of a protein that can be recruited through its SH2 domains to immunoreceptor tyrosine-based inhibitory motifs (ITIMS), but has defective phosphatase catalytic activity. Binding of this inactive protein to SH2 domain docking sites and substrates effectively blocks recruitment of endogenous wild-type SHP-1 (361), resulting in inhibition of SHP-1 function.

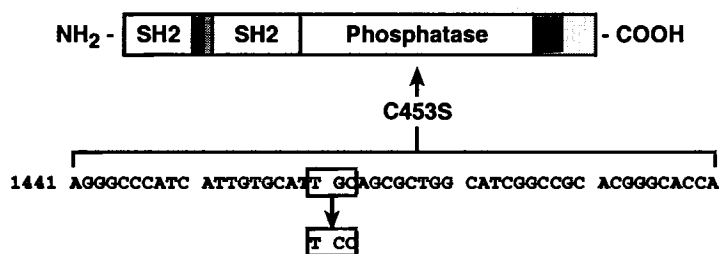


Figure 4.1. The dominant negative SHP-1 mutation C453S. The G → C transversion changes the essential catalytic cysteine (TCG), to a serine (TCC), inhibiting the phosphatase catalytic activity of the SHP-1 protein. This catalytically inactive form of SHP-1 competes for binding sites with wild type SHP-1, effectively reducing the activity of the wild type protein.

To direct expression of the SHP-1 C453S mutant to macrophages, the promoter for *c-fms* was used. The *c-fms* gene encodes the cell surface receptor for macrophage colony stimulating factor (CSF-1) (362, 363). CSF-1 is a cytokine required for the proliferation and survival of cells in the mononuclear phagocyte lineage. Expression of the *c-fms* encoded CSF-1 receptor (CSF-1R) is regulated by tissue specific promoters (364). Expression occurs on macrophages as well as in the placenta during gestation. The promoter used in these studies induces macrophage specific expression of CSF-1R (365).

Materials and Methods

Construction of SHP-1 dominant negative transgene for expression in macrophages.

The pBluescript plasmid containing wild-type SHP-1 cDNA with a KT3 epitope tag and human growth hormone (hGH) poly A sequence driven by the CD11b promoter was a gift from Dr. Taolin Yi (Cleveland Clinic Foundation, Cleveland, OH). The macrophage specific *c-fms* (CSF-1R) promoter, (365) (in pBluescript) was a gift from Dr. Alessandra D'Azzo (St. Jude Children's Research Hospital, Memphis, TN). The SHP-1-KT3-hGH segment of the SHP-1 construct was excised using *EcoRI* and was subcloned into a p138 plasmid linearized with *EcoRI*. The *c-fms* promoter was excised from pBS with *SpeI* and *EagI* and ligated into the p138 plasmid containing the SHP-1-KT3-hGH construct, which was linearized with *XbaI* and *NotI*. The QuikChange Site-directed mutagenesis kit (Stratagene) was used to create a G to C transversion which changed the essential catalytic cysteine to serine, producing a construct that will express a putative dominant negative SHP-1 protein (359). Mutagenesis was performed according to manufacturers instructions using the following primers: (5'-CCG ATG CCA GCG CTC GAA TGC ACA ATG ATG G-3') and (5'-CCA TCA TTG TGC ATT CCA GCG CTG GCA TCG G-3').

Transgenic mice

Transgenic C57BL/6J mice were generated by standard methods by The Jackson Laboratory's microinjection service. Potential founders were screened for the transgene by PCR using primers specific for the second intron of the *Hcph* sequence (5'-TCC CTG GGA GCT TCC TGG CTC-3' and 5'-GGT GGG GTC CGA GCA GTT CAG-3').

Amplification with the endogenous SHP-1 gene yields a product of 494 bp, while amplification with transgenic SHP-1 yields a product of 251 bp. Presence of the transgene was confirmed using primers for human growth hormone (5'-CAT CCC TGT GAC CCC TCC-3' and 5'-CTC CAA ACC ACC CCC CTC-3').

Hematology

Blood was collected from the retro-orbital sinus with EDTA coated capillary tubes and placed directly into Isoton II diluent (Coulter). Hematologic values were determined using the ADVIA 120 hematology system (Bayer Corp, Tarrytown, NY). Control C57BL/6J mice were age and sex matched with each SHP-1 C453S transgenic animal tested.

Culture of bone marrow macrophages

To expand bone marrow macrophage populations for analysis, bone marrow plugs from femurs and tibias of transgenic and wild type mice were harvested by extrusion with cold HBSS (Sigma). Marrow plugs were disrupted by passage through a 25g needle. The resulting single cell suspensions were washed 2x in sterile HBSS, counted with a model ZBI Coulter counter. Cells were resuspended in complete media (RPMI-1640; Sigma, containing 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin and 50 µM 2-β-mercaptoethanol) containing 500 units/ml recombinant human (rHu) CSF-1 (Cetus). A total volume of 5 ml CSF-1 supplemented medium containing 10⁶ cells/ml was added to 25 cm² tissue culture flasks. Cultures were incubated at 37°C in 5% CO₂ for 7 days with periodic changes of media, maintaining the

appropriate CSF-1 concentrations. Cells were lysed in the flask for western blotting as described.

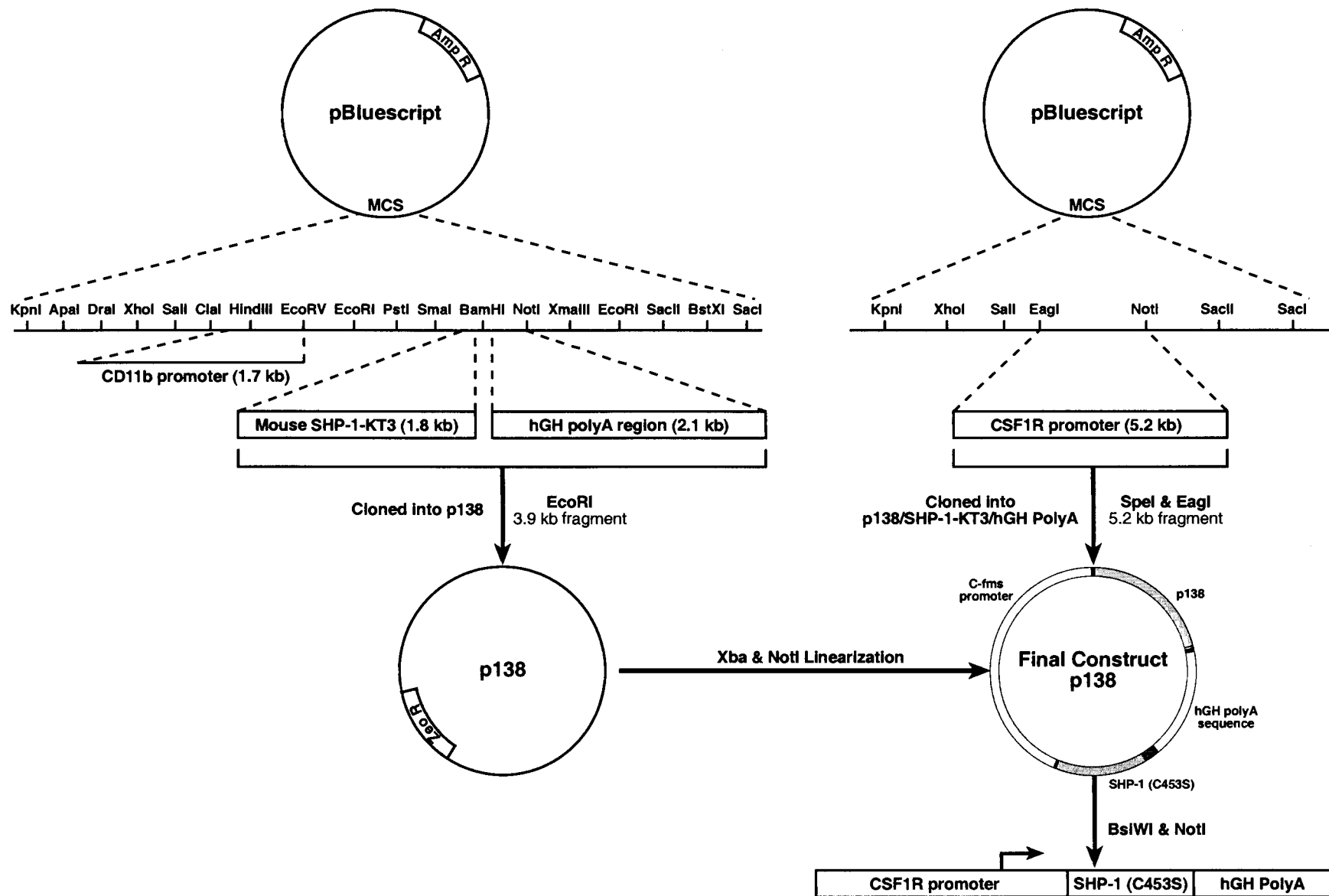
Immunoblotting

Cultured cells were lysed for 30 minutes at 4°C in cold lysis buffer (50 mM Tris, pH 8.2, 150 mM NaCl, 1% (Octylphenoxy)polyethoxyethanol (Igepal CA-630; Sigma), plus 1 tablet Complete, Mini Protease Inhibitor Cocktail Tablet w/EDTA (Roche Molecular Biochemicals) / 10 ml buffer. Cell lysates were clarified by centrifuging for 20 min at 10,000g at 4° C. Protein levels were measures using the DC Protein Assay (Bio-Rad, Hercules, CA). 20 µg of each sample was resolved in a 12 % SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Bedford, MA). The membranes were blocked in 5% dry milk overnight at 4° C, then incubated with rabbit anti-mouse SHP-1 for 1-2 hours at room temp, with shaking. Membranes were then washed 3 X 5 min with TBS-T (TBS w/ 0.05% Tween 20), incubated with HRP conjugated goat anti-rabbit Fab² at RT for 30 – 60 min with shaking, then washed 5 x 5 min in TBS-T. Specific antibody signals were detected using an enhanced chemiluminescence kit (ECL^{plus}, Amersham Pharmacia Biotech, Uppsala, Sweden).

Results and Discussion

The transgene was constructed as described (Figure 4.2). Sequencing from the carboxy terminal end of SHP-1 through to the hGH poly-A sequence revealed an error in the original construction of the CD11b/SHP-1-KT3/hGH plasmid.

Figure 4.2. Construction of SHP-1 C453S transgene. Murine SHP-1-KT3/hGH poly A was excised from pBluescript with EcoRI and cloned into p138, linearized with EcoRI. The CSF-1R promoter was excised from pBluescript with SpeI and EagI and was cloned into the p138 plasmid containing the SHP-1-KT3-hGH construct, which had been linearized with XbaI and NotI. Mutagenesis was performed on the final construct to change the essential catalytic cysteine to a serine (see Figure 4.1). The resulting transgene should express a dominant negative SHP-1 protein under control of the macrophage-specific promoter of CSF1-R (*c-fms*).



A fragment of SHP-1 consisting of base pairs 88 – 266 of the wild type protein was present at the carboxyl terminal end of the SHP-1 transgene, apparently in place of the KT3 epitope tag, since a KT3 sequence was not found. However, the full coding sequence of the SHP-1 gene was present. Absence of the KT3 tag raises a number of challenges when trying to identify the transgenic protein in expression studies. Studies using dominant negative proteins that retain structural similarity to their wild type counterparts rely on an epitope tag to identify the transgenic protein by immunoblotting (359, 360, 366). The SHP-1 C453S transgenic protein differs from the wild type SHP-1 protein by only one amino acid, and the *c-fms* promoter drives expression of the transgene in cells that normally exhibit significant expression of endogenous SHP-1. Without an epitope tag, demonstration of the successful expression of the transgene will rely on an increase in SHP-1 expression levels in macrophages, altered phosphorylation on tyrosine residues of SHP-1 substrates or functional studies.

Transgenic mice were made by conventional methods, and founders were identified by PCR as described (Figure 4.3). From the first 34 possible founders, 4 females were identified as transgenic. Of the following 71 possible founders, none tested positive for the transgene. A new batch of transgene DNA was submitted to the institutional microinjection service for generation of additional transgenic mice. The 4 founders were mated to C57BL/6J males. One female founder died unexpectedly at 12 weeks of age, having produced no offspring. Founder #6 and founder #25 have produced offspring. The first litter of each of these founders contained transgenic mice. All existing transgenic mice, both founders and offspring from first litters, were bled from the retro-orbital sinus for peripheral blood analysis.

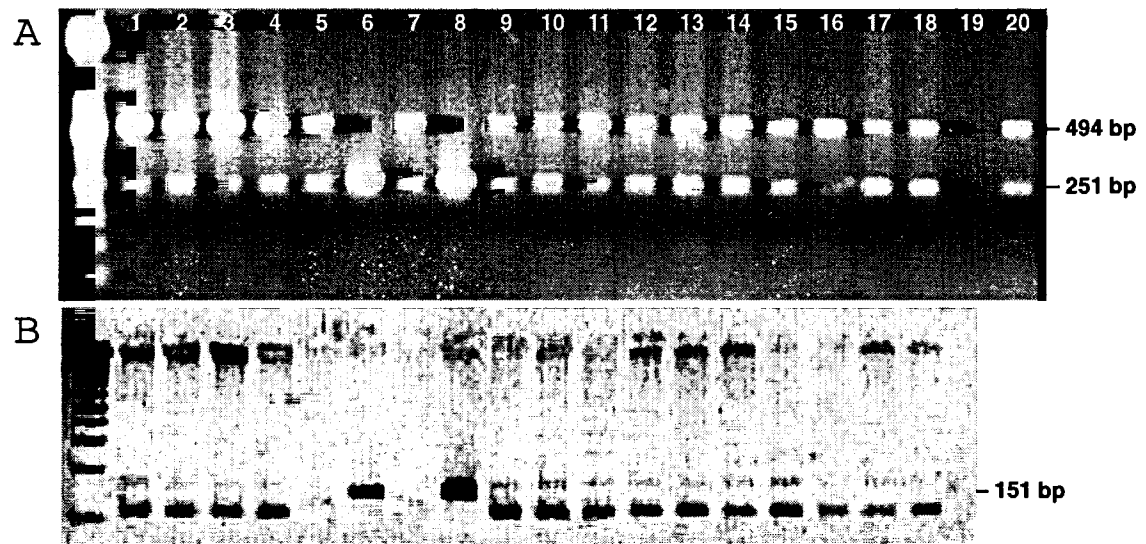


Figure 4.3. PCR typing of SHP-1 C453S transgenic founders. Lane numbers correspond to both upper and lower panels. A) Typing using primers for SHP-1. Lanes 6 and 8 show the intense 251 bp band indicating presence of the transgene. Lane 19: dH₂O, lane 20: wild type DNA control. Amplification of a 251 bp band in wild type mice was unexpected, so transgene presence was confirmed by further typing with primers for hGH poly-A sequence. B) Typing with primers for hGH results in amplification of a 151 bp band in mice carrying the SHP-1 C453S transgene (Lanes 6 and 8).

If the transgene was functional, and the SHP-1 C453S protein had a true dominant negative effect, one would expect to detect an increase in percentages of myeloid lineage cells in peripheral blood of transgenic mice. However, peripheral blood counts of transgenic and non-transgenic mice were not significantly different (Figure 4.4). There

was no significant variation amongst mice carrying the transgene, so data from all transgene positive mice were pooled for comparison to wild type.

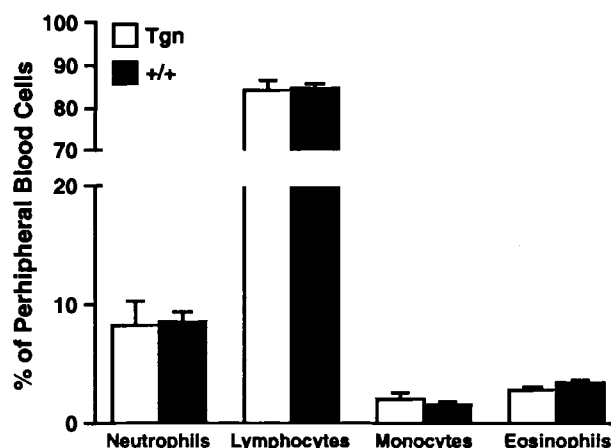


Figure 4.4. Peripheral blood analysis of SHP-1 C453S transgenic mice vs. wild type C57BL/6J mice. (n = 8)

Macrophages containing numerous functional copies of the SHP-1 C453S transgene should produce greater total levels of SHP-1 protein than wild type cells. To examine SHP-1 expression levels in the cell population to which transgene expression was directed, one transgenic offspring from founder #25 was sacrificed. Bone marrow was collected from femurs and tibias and cultured with CSF-1 to expand bone marrow macrophage populations. Analysis of these cultured cells by western blotting showed no increase in SHP-1 expression between SHP-1 C453S and wild type cells (Figure 4.5). These data suggest that the transgene is not functional in bone marrow macrophages grown in the presence of CSF-1. However, without an epitope tag to distinguish between SHP-1 C453S and endogenous SHP-1, it is possible that the transgene is being expressed,

but does not result in a detectable increase in SHP-1 protein. However, there was also no detectable difference in tyrosine phosphorylation levels in SHP-1 C453S bone marrow macrophages versus wild type (Figure 4.6). This suggests that the presence of the transgene does not result in alteration of SHP-1 phosphatase function.

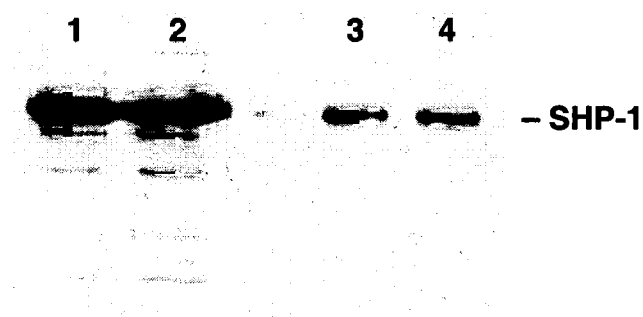


Figure 4.5. Anti-SHP-1 western blot on bone marrow macrophage and spleen cell lysates from a SHP-1 C453S transgenic and control mouse. Lanes 1 & 2 contain lysates from bone marrow macrophages, lanes 3 & 4 contain total spleen lysates. Lanes 1 & 3 are SHP-1 C453S Tgn, lanes 2 & 4 are wild type. 2 μ g of protein/lane.

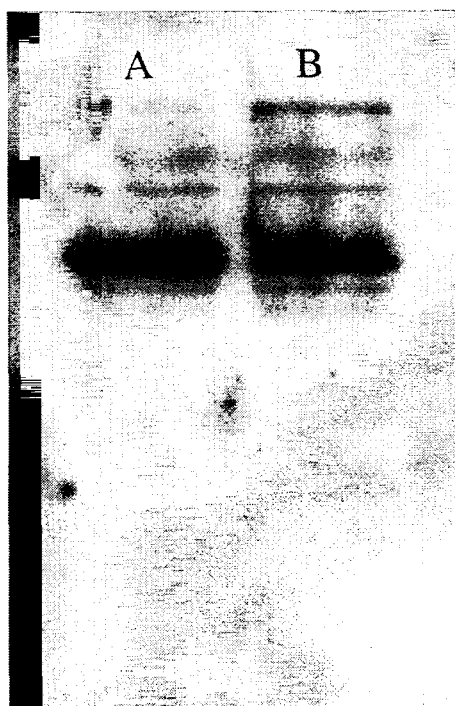


Figure 4.6. Anti-tyr western blot on bone marrow macrophages of A) SHP-1 C453S transgenic mice and B) C57BL/6J control mice.

Preliminary studies of mice carrying a transgene for macrophage-specific SHP-1 C453S expression do not confirm a functional effect of the transgene. Our collaborator (Dr. Taolin Yi) has recently developed a novel, more effective dominant negative SHP-1 mutant. Although we will continue to expand the current founder colonies of SHP-1 C453S, we will investigate the efficacy of this new dominant negative SHP-1 mutant for use in any future studies using a transgenic approach to alter SHP-1 function in a cell specific fashion.

Chapter 5

ESTABLISHMENT AND CHARACTERIZATION OF A NEW CELL LINE (MOS-J) FROM A SPONTANEOUS C57BL/6J MOUSE OSTEOSARCOMA.³

Abstract

This chapter describes the establishment and characterization of an osteoblast-like cell line derived from a spontaneously occurring chondroblastic osteosarcoma in a C57BL/6J mouse. The tumor line, MOS-J, forms solid tumors when injected intramuscularly into immunocompetent syngeneic hosts, mimicking endochondral bone development. These transplantable tumors have the capacity to destroy and invade existing bone, and invade vessels in close proximity to the tumor. In culture, MOS-J cells form layers of pleomorphic cells with high mitotic activity. These cells have marked alkaline phosphatase enzyme activity and form calcified foci *in vitro* that stain with alizarin red. MOS-J cells also promote osteoclast development from normal bone marrow. These characteristics indicate the potential utility of the MOS-J osteosarcoma cell line as a model for the studies of human osteosarcoma and normal bone biology.

Introduction

In an effort to substantiate the role of SHP-1 as a tumor suppressor gene, our lab maintains aging stocks of *+/me* and *+/me^v* mice, monitoring tumor incidence, and classifying and collecting samples of each neoplasm for analysis. A 25-month-old female *+/me^v* mouse that was part of this study developed a firm, palpable mass in the

right distal femur. The mass was identified histologically as a chondroblastic osteosarcoma, a rare tumor in mice (367, 368). Preliminary investigation of this tumor did not support the hypothesis that it resulted from loss of SHP-1 heterozygosity. However, the cell line derived from this tumor may have significant value as a model of human osteosarcoma.

Osteosarcoma is a malignancy of bone that commonly affects adolescents and young adults, accounting for approximately 20% of all primary malignant neoplasms of the bone in humans (369). Treatment often includes amputation of the affected area and adjuvant chemotherapy treatment. Despite aggressive surgical and medical therapies, 5-year survival rates range from only 10 to 60 %.

Cell lines provide an important resource for investigating the pathophysiology of many types of cancers. These lines can be used for *in vitro* study of the biological behavior of cancer cells, including the basic mechanisms of oncogenesis, expression of growth factors and receptors and assessment of cell sensitivity to anti-neoplastic agents. Mouse cancer cell lines provide a valuable tool for *in vivo* study of *in situ* and metastatic tumor cell behavior following injection of the cells into immunocompetent syngeneic hosts or into immunodeficient mice.

Here we describe an osteosarcoma cell line derived from a primary spontaneous mouse chondroblastic osteosarcoma (osteochondrosarcoma). This cell line has many characteristics of osteoblast-like cells, and may be a useful model for the study of both osteosarcoma and normal bone biology, both *in vivo* and *in vitro*.

³ Modified from: Joliat MJ, Lyons BL, Umeda S, Lynes MA, Shultz LD. Establishment and characterization of a new cell line (MOS-J) from a spontaneous C57BL/6J mouse osteosarcoma. In prep.

Materials and Methods

Source of cancer tissue

The MOS-J (Mouse Osteogenic Sarcoma – Jackson Laboratory) cell line was derived from a spontaneous chondroblastic osteosarcoma that arose in the right distal femur of a 757-day old female C57BL/6J mouse heterozygous for the viable motheaten (*Hcph^{mev}*, hereafter abbreviated *me^v*) mutation. The mouse was euthanized by CO₂ asphyxiation and the lesion was removed for examination. Minced pieces of the tumor were placed subcutaneously into BALB/cByJ-*Prkdc^{scid}/Prkdc^{scid}* (hereafter called *scid*) mice. Additionally, portions of the tumor were disaggregated into a single cell suspension and cultured to establish the cell line. Other sections of the tumor were fixed for histological analysis as described below.

Histopathology

Tissues were fixed and demineralized in Bouin's solution, or fixed in Fekete's acid alcohol formalin and demineralized using Cal-EX decalcifying solution (Fisher Scientific, Fair Lawn, NJ). Tissues were then embedded in paraffin, sectioned at 6 µm and stained with Mayer's hematoxylin and eosin (H&E) for histological examination. Immunohistochemistry was carried out on serial tumor sections using mouse anti-alpha-smooth muscle actin (Sigma) for identification of vessel wall invaded by tumor cells. Non-specific staining was blocked on deparaffinized sections with peroxidase blocking reagent (DAKO). Tissue sections were then incubated in primary antibody for 1 hour at room temperature in a humidified chamber, washed 3 x 5 minutes in PBS and then incubated with biotinylated secondary antibody against mouse IgG2a isotype for an

additional hour. After another three 5-minute washes in PBS, tissues were incubated in avidin/horseradish-peroxidase conjugate for 45 min, followed by an additional trio of 5-minute washes. Tissues were then incubated in diaminobenzidine (DAB) for 5 minutes at room temperature and washed in tap water for 5 to 15 min. Tissues were counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Processing of tissues was conducted by The Jackson Laboratory's Biological Imaging service.

Establishment of the MOS-J cell line

Immediately after removal of the original neoplasm, normal muscle, vascular tissue, fascia, and fat were removed from the specimen and discarded. Fractions of the tumor were minced and dispersed in complete media (cRPMI: RPMI-1640 (Sigma, St. Louis, MO) containing 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin and 50 µM 2-β-mercaptoethanol) in a 50 ml centrifuge tube. The resulting single cell suspension was seeded in 25 cm² tissue culture flasks and incubated at 37° C in humidified 5% CO₂. Culture medium was replaced twice a week until cells reached confluence. Cultures were exposed to trypsin-EDTA to release adherent cells from the surface of the flask, then were washed with fresh medium and subcultured at a density of 2×10^5 cells/ml under similar culture conditions.

Growth rate

To determine the rate of cell growth, cells were plated at 5×10^3 cells/cm² in 35 mm 6-well plates in minimum essential media containing 10% FBS. Each well had a

growth area of 9.4 cm². At 2, 3, 4, 5, 6, and 7 days of incubation, cells were trypsinized and counted with a hemacytometer. Values are expressed as the mean number of cells \pm SEM from six wells.

Cell culture characteristics

Cells from the parental line were maintained in cRPMI. For studies of the characteristics of cells in culture, cells were seeded in a culture dish containing several glass coverslips. At 2, 5 and 9 days, a single coverslip was removed from the culture. Cells were fixed in methanol for 15 minutes, then stained with H&E, dehydrated through a series of ethanols to xylene and mounted on a slide with Permount (Fisher).

Alkaline phosphatase detection *in situ*

Cells were plated into 6-well culture dishes (Corning Costar, Corning, NY) at 5×10^5 cells per ml in a total volume of 6 ml complete media per well. They were maintained in a humidified, 5% CO₂ incubator at 37° C for 72 hours. NIH 3T3 cells were plated simultaneously and used as a negative control. After 72 hours at 37° C, the culture dishes were placed on ice. The medium was removed and the cells were washed twice in cold PBS. Cells were then fixed in the dishes for 20 min on ice with methanol. The methanol was removed and cells were washed twice in PBS. A BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) substrate kit for alkaline phosphatase was used (Zymed, San Francisco, CA) to detect alkaline phosphatase activity. The substrate solution was prepared as directed by the manufacturer and 5 ml was added to each well. Color development was allowed to occur for 5 - 10 minutes at room

temperature, after which the substrate was removed and the cells were washed twice in distilled H₂O.

Alizarin red staining

Cells were grown on coverslips in a culture dish for 30 days in cRPMI. Presence of calcium was assessed in cultured cells using a Dahl's method for calcium (370), with modifications. Briefly, cells were fixed for 15 min in methanol, then air-dried. Cells were then incubated for 10 minutes in a solution of 1% aqueous alizarin red, which had been adjusted to a pH of 6.4 with 0.1% ammonium hydroxide. Cells were rinsed three times in dH₂O, counterstained in 0.2% light green for 15 seconds, and rinsed briefly in dH₂O. Finally, cells were dehydrated through a graded series of alcohols to xylene and mounted using Permount (Fisher).

Support of osteoclastogenesis

C57BL/6J bone marrow cells ($2.5 \times 10^4/\text{cm}^2$) were seeded simultaneously with tumor cells ($1 \times 10^4/\text{cm}^2$) in 8 well LabTek chambers and 6 well plates, in RPMI-1640 (Sigma) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-β-mercaptoethanol, supplemented with 10 nM/L 1-α-25 dihydroxy (OH)₂ Vitamin D₃ (Calbiochem, San Diego, CA). Cells were grown both with and without 500 U/ml recombinant human (RHu) CSF-1 (Cetus, Emeryville, CA) added to the medium to promote osteoclast growth. As a control, MOS-J cells and bone marrow cells were each grown alone with CSF-1. Cells were stained for tartrate resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma).

Oncogenicity in mice

Five male mice of each of the three strains (C57BL/6J, C57BL/6J-*Rag-1*^{-/-}, and NOD-*Rag-1*^{-/-}) were injected in the quadriceps muscle with cultured tumor cells (1.3×10^5 cells/0.1ml sterile PBS). Growth was assessed by radiographic examination at 2 and 6 weeks post inoculation. At seven weeks after injection, the primary mass was removed from two mice in each group by aseptic surgical amputation of the affected limb, as described below (Dr. B. Lyons). The remaining mice of each strain were euthanized and necropsied. Grossly evident nodules and selected organs were prepared for histological examination as previously described. Mice surviving amputation were assessed by radiographic examination for evidence of recurrence or metastasis.

Transmission electron microscopy

Ultrastructural studies were performed on specimens obtained from transplanted neoplasms in BALB/cByJ-*scid* mice. Neoplasms were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 60 minutes. Samples were rinsed in 0.1 M cacodylate buffer for 15 minutes, then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 15 minutes, dehydrated in a graded series of ethanols (50 – 100%), then passed through polypropylene oxide as the intermediate solvent. Samples were re-embedded in 1:2 propylene oxide / Epon mixture for 4 hours, exposed to a 1:3 propylene oxide / Epon mixture, then transferred to a conical Beem capsule. One hour later, the cells were placed in a dessicator and incubated overnight, then polymerized in a 60° C oven for 48 hours. One to two mm sections were cut and stained with Toluidine blue and ultrathin (70 – 90nm) sections prepared from selected areas were mounted on copper grids. The sections

were stained with aqueous uranyl acetate and Reynold's lead citrate and were examined using a transmission electron microscope (371).

Amputation of affected limb

Coxofemoral disarticulation (372) was performed to amputate the affected leg. Mice were anesthetized using Avertin (250 mg/kg body weight). Muscle and skin layers were closed separately with absorbable sutures and the mice were kept in a quiet, heated environment and monitored until ambulatory. This procedure was conducted by a licensed veterinary surgeon, (B. Lyons, D.V.M.). The protocol was approved by the institutional Animal Care and Use Committee (ACUC).

Results

Histopathology of the original tumor

The original tumor occurred spontaneously in the right distal femur of a 25-month-old C57BL/6J *+/*me*^v* female mouse. The mass was lobular in shape, firm and pale white in color. Histological evaluation showed variably sized bundles and streams of closely packed undifferentiated spindled cells with discernable cell borders, containing numerous mitotic figures. These cells contained a moderate quantity of basophilic, finely fibrillar cytoplasm. Nuclei were pleomorphic, oval to elongate, containing coarse and finely granular heterochromatin. Scattered throughout the mass were areas of irregular, often interconnecting trabeculae of osteoid, generally contiguous with large areas of well-differentiated hyalin cartilage. Numerous multi-nucleated giant cells resembling osteoclasts were observed within the trabecular regions of the neoplasm. This tumor was

interpreted to be a well-differentiated osteochondrosarcoma. No metastases were observed in lung, liver or other organs.

Cell culture characteristics

Growth rate and morphology of MOS-J cells were examined in culture. Figure 5.1 shows the growth rate of MOS-J cells in MEM with 10% FBS. Under growth assay conditions in 9.4 cm² wells, cells began to detach after 7 days. However, in 75 cm² culture flasks, cultures have been maintained successfully without passage for > 2 months with bi-weekly media changes. Cultured MOS-J cells form layers of large, closely-spaced, polygonal and elongated cells with random orientation, containing pleomorphic nuclei and abundant cytoplasm (Figure 5.2). Multiple mitotic figures are present as well as individual cell necrosis characterized by pyknotic and karyorrhectic debris.

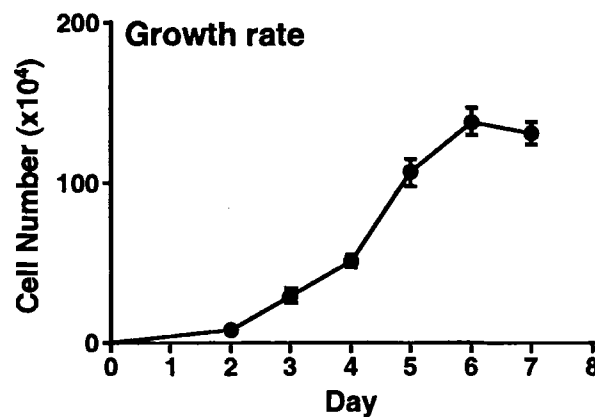


Figure 5.1. Growth rate of MOS-J cells in culture.

Alkaline phosphatase activity

Alkaline phosphatase activity is a characteristic feature of osteogenic differentiation of osteoblast-like cells (373). MOS-J cells showed marked alkaline phosphatase activity by enzyme cytochemistry (Figure 5.3 A). Negative control NIH 3T3 cells under similar conditions showed no activity (data not shown).

Alizarin red staining

Alizarin red detects calcium deposition, which is a hallmark of ossification. Cells grown in culture contained numerous foci that stained positive for alizarin red (Figure 5.3 B, C). In the areas of darkest red staining, cells showed a circular pattern of aggregation.

Osteoclast growth assay

MOS-J cells promoted osteoclast growth in normal bone marrow cells (Figure 5.3 D). TRAP-positive cells were present in cultures containing MOS-J cells with normal bone marrow cells. No quantifiable difference was seen in numbers of TRAP-positive cells in cultures grown with or without CSF-1. No TRAP positive cells were seen in CSF-1 supplemented cultures of tumor cells alone or bone marrow cells alone (Figure 5.3 E, F).

Oncogenicity *in vivo*

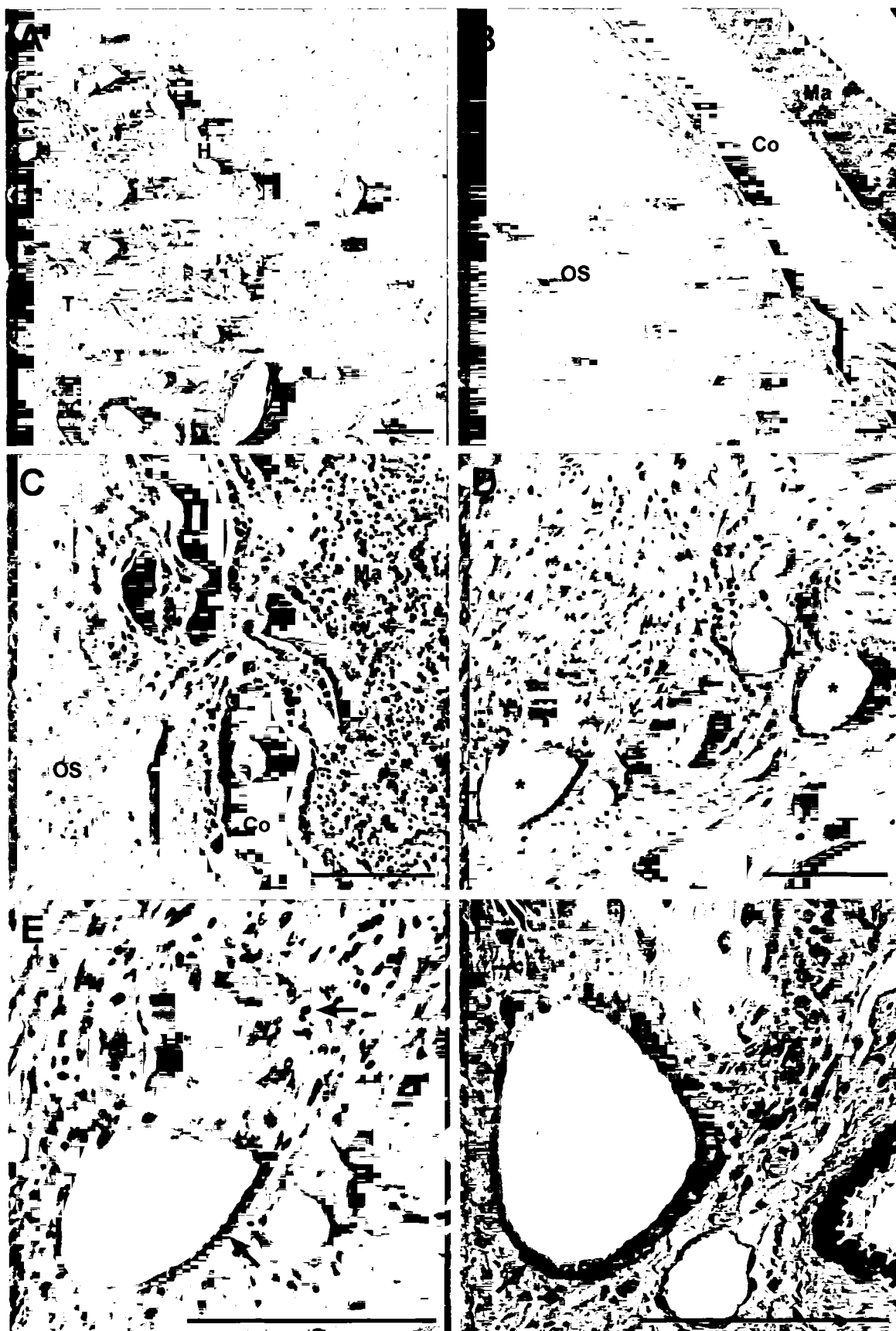
MOS-J cells form rapidly growing tumors when injected IM into immunodeficient mice as well as in immunocompetent syngeneic hosts. At two weeks post injection, radiodense lesions were visible in the muscle of all mice in the vicinity of injection. In some cases, osteolysis was evident in the existing bone adjacent to the lesion (Figure 5.4 B). At 7 weeks post injection, lesions were prominent, radiodense, and involved the bulk of the adjacent bone (Figure 5.4 C). In all cases, the injection ultimately resulted in one visible, palpable mass that was firm and occasionally multi-lobated.

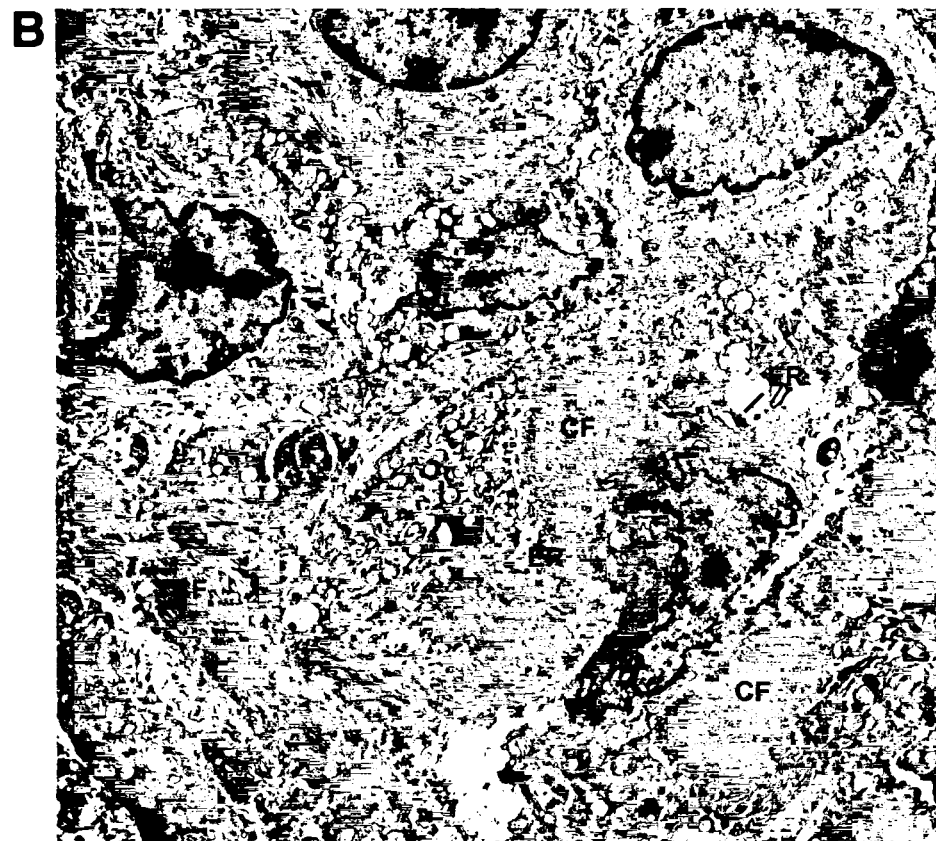
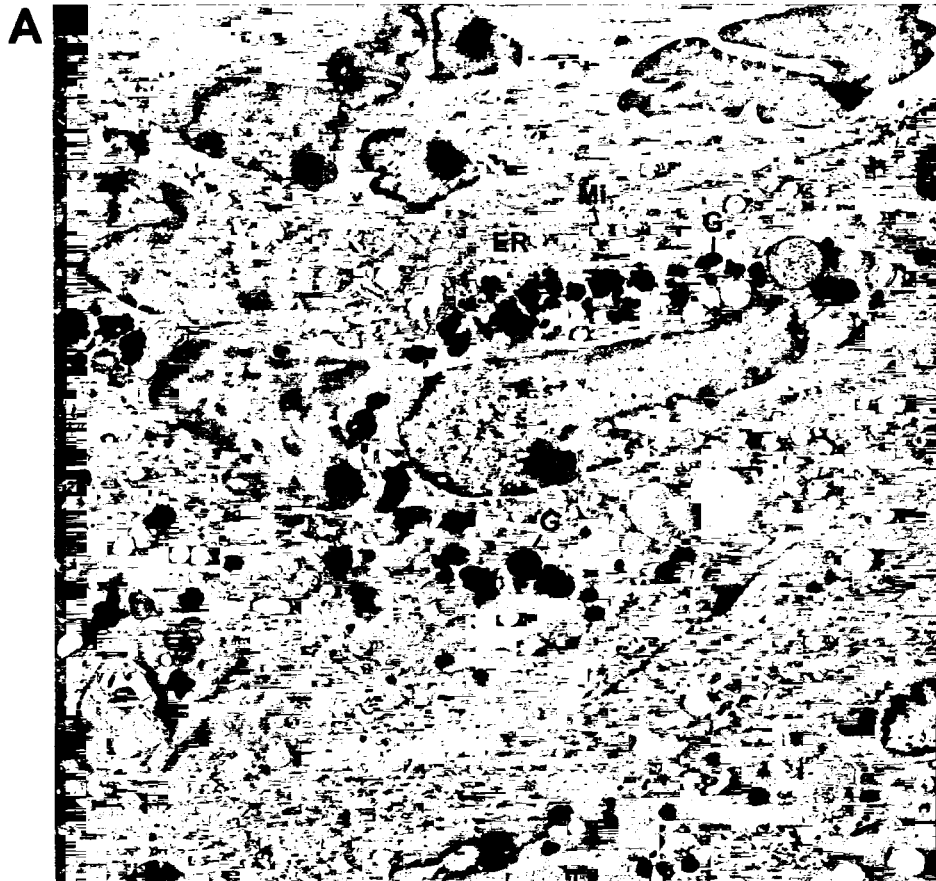
Histologically, the neoplasms were generally fairly well demarcated and non-encapsulated, with cells on the perimeter resembling periosteum. Spindle-shaped cells containing areas of osteoid trabeculae were interspersed with cartilaginous matrix (Figure 5.5 A). Basophilic chondroblasts were visible in areas of cartilage production. Regions of well-mineralized neoplastic bone as well as reactive normal bone were present (Figure 5.5 A, B). Lytic lesions were evident in existing bone (Figure 5.5 C). With IM injection, there was no evidence of metastasis, radiographically or histologically, even after amputation of the affected limb. However, there were multiple sites within individual tumors where neoplastic cells were seen in close proximity to or even penetrating or obliterating the vessel walls and growing into the lumen of vessels (Figure 5.5 D, E, F).

Ultrastructural findings of transplanted tumors

Tumor cells include large, irregularly shaped nuclei containing margined chromatin and prominent nucleoli (Figure 5.6). The cytoplasm contained abundant rough

endoplasmic reticulum (rER) and mitochondria. In some areas of the tumor, irregular electron dense granules resembling mineral deposits formed a halo-like pattern around the nucleus in the cytoplasm (Figure 5.6 A). Other areas of the tumor were characterized by an abundance of extracellular collagen fibrils (Figure 5.6 B).





Discussion

The MOS-J cell line was derived from a spontaneous chondroblastic osteosarcoma in a C57BL/6J $+/me^v$ mouse. The me^v mutation disrupts the structural gene (*Hcph*) encoding SHP-1 protein tyrosine phosphatase, also referred to as hematopoietic cell phosphatase. The tumor derived MOS-J cell line was first examined for loss of heterozygosity of the wild type *Hcph* gene as part of a study of the putative role of *Hcph* as a tumor suppressor gene (254). If SHP-1 does indeed function as a tumor suppressor gene, increased tumor rates would also be expected in heterozygous $+/me$ or $+/me^v$ mice. Tumors would be expected to arise from cell types in which SHP-1 is known to function as a negative regulator, such as T, B, or myeloid cells, and these neoplastic cells would be expected to have deficient SHP-1 expression. The discovery of a firm tumor associated with the bone was unexpected in this study and was of particular interest. However, preliminary studies do not support the possibility that this tumor was caused by loss of expression of the SHP-1 protein (data not shown). Moreover, the cell line derived from this tumor has an osteoblast-like phenotype, and it has been shown that normal osteoblasts do not express SHP-1 (374). Nonetheless, many characteristics of the MOS-J cell line suggest that it may have utility in the study of bone biology as well as osteosarcoma biology and treatment.

The MOS-J cell line has an aggressive phenotype characterized by unrestricted growth, but accompanied by numerous functional and histocytological features of normal osteoblasts. Alkaline phosphatase enzyme activity, ossification both *in vitro* and *in vivo*, and ability to promote osteoclast development in culture are all indicative of osteoblast-like function by the MOS-J tumor cells. These transplanted tumor cells mimic the

process of endochondral ossification *in vivo*. Presence of basophilic chondroblasts and cartilaginous matrix within the tumor is similar to the histological variant of the human conventional osteosarcoma described as chondroblastic osteosarcoma.

The derivation of this cell line from a spontaneous tumor in a C57BL/6J mouse is a particular advantage over osteosarcoma cell lines derived from other species, less common mouse strains, or mice in which oncogenesis was promoted by insertion of an oncogenic transgene. These cell lines are often limited to transplantation into immunocompromised hosts. Certain transgenes that induce oncogenesis, such as the SV40 large-T antigen, can be immunogenic (375, 376). Transplantation of cells derived from transgene induced tumors can result in tumor rejection by immunocompetent syngeneic hosts. Conversely, MOS-J cells may be transplanted, without rejection, into immunocompetent C57BL/6J mice. Many strains of mice carrying targeted mutations or transgenes related to bone and cancer biology are maintained on the C57BL/6J background (see (265)). Study of the behavior of MOS-J cells transplanted into mice bearing one or more of these mutations may provide insight into important biological interactions involved in tumor growth and metastasis or bone development and maintenance.

MOS-J cells have many histocytological features of normal osteoblasts. They support osteoclast development when cultured with normal bone marrow, suggesting that these cells may be used in the study of intercellular interactions or the investigation of factors produced by osteoblasts that control osteoclast differentiation and activation. CSF-1 is essential for normal osteoclast development (377). The observation that MOS-J cells promote osteoclast formation *in vitro* without the addition of exogenous CSF-1

suggests that the MOS-J tumor cells, like normal osteoblasts (378), secrete CSF-1.

Experiments using other osteosarcoma cell lines have demonstrated that normal osteoblasts secrete Interleukin-11 (IL-11) (379), and that hepatocyte growth factor (HGF) can induce secretion of IL-11 from osteoblast-like cells (380). IL-11 stimulates TRANCE (TNF-related activation-induced cytokine) expression on osteoblasts (381), which is crucial to the stimulation of osteoclast differentiation and bone resorption (375). The MOS-J cell line may be a valuable tool in further understanding the intricate processes involved in skeletal development and restructuring.

Although evidence suggests that the neoplastic characteristics of MOS-J cells are not associated with mutations in SHP-1, further molecular characterization of the MOS-J cell line has not been conducted. There are a large number of genetic mutations known to be associated with osteosarcoma in humans (for review, see (382)). Investigation into the genetic abnormalities existing in this line may identify novel mutations that lead to the development of osteosarcoma, or may reveal mutations already known to be associated with human osteosarcoma, providing a direct model for the study tumor development.

Transplanted MOS-J cells have the capacity to infiltrate adjacent bone following intra-muscular injection, and appear to invade vessels at multiple sites within transplanted tumors. However, metastases have not been found in other organs. It has recently been shown that intra-tibial injection of the rat osteosarcoma cell line UMR 106-01 into athymic mice can result in lung metastasis where alternate methods of injection using the same cell line have failed to produce metastasis (383). A number of genetic variations have been associated with metastatic potential in an osteosarcoma model system (384).

Isolation of a metastatic variant of MOS-J or development of an injection system that promotes metastasis of the transplanted tumor would enhance the value of this cell line for the study of genetic alterations associated with metastasis. However, we have assessed the metastatic potential of MOS-J cells following intra-tibial injection, and have found no evidence of metastasis. Despite the fact that a metastatic variant of MOS-J has not yet been identified, MOS-J cells may be valuable in comparative gene expression studies with osteosarcoma models that do show evidence of metastasis, such as the histologically and behaviorally distinct tumors that arise spontaneously in SV40 Tag transgenic mice (385, 386).

CONCLUSION

Mice with spontaneous genetic mutations have provided crucial tools with which to develop our knowledge of normal and pathological biological processes. Studies using mice carrying the motheaten or viable motheaten mutations have advanced the understanding of numerous signaling pathways in the immune and hematopoietic systems. Recently, SHP-1 has received significant attention for its role in development of human cancers (261, 262, 264, 387) and for its contribution to other diseases of hematological and immunological dysregulation (263, 388). Continued investigation into the extensive signaling capacities of SHP-1 may lead to further insights into these diseases and into the complex physiological processes involved in maintaining homeostasis of the immune system.

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1. Lyons BL, Lynes MA, Hadjout N, **Joliat MJ**, Goldwasser E, Greiner DL, Lawrence DA, Shultz LD. The mechanisms of anemia in SHP-1 protein-tyrosine phosphatase-deficient “viable motheaten” mice. In prep.

2. **Joliat MJ**, Lyons BL, Umeda S, Lynes MA, Shultz LD. Establishment and characterization of a new osteogenic cell line (MOS-J) from a spontaneous C57BL/6J mouse osteosarcoma. In prep.
3. Christianson SW, Greiner DL, DeLuca D, Leif J, Phillips NE, Hayes SM, Hayashi SI, **Joliat MJ**, Lyons BL, Shultz LD. T cell developmental defects in “viable motheaten” mice deficient in SHP-1 protein-tyrosine phosphatase. *J. Autoimmun.* In press.
4. **Joliat MJ**, Lang PA, Lyons BL, Lynes MA, Yi T, Sundberg JP, Shultz LD. Absence of CD5 dramatically reduces progression of pulmonary inflammatory lesions in SHP-1 protein-tyrosine phosphatase-deficient viable motheaten mice. *J. Autoimmun.* In press.
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Abstracts

10. Lyons BL, Lang PA, **Relyea MJ**, Sundberg JP, Shultz LD. 2000. The role of granulocyte colony-stimulating factor in the development of immunopathologic changes in viable motheaten mice. (Abstract) *Vet. Path.* 37:542.

11. **Relyea MJ**, Lang PA, Lyons BL, Sundberg JP, Shultz LD. 2000. The role of CD5 on immunopathologic changes in viable motheaten mice. (Abstract) *FASEB J* 14:6, A999-52.36.

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