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Characterization of the pathophysiology of Wiskott-Aldrich transgenic mice

Sharon Golda Witonsky

To the Graduate Council:

I am submitting herewith a dissertation written by Sharon Golda Witonsky entitled "Characterization of the pathophysiology of Wiskott-Aldrich transgenic mice." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

J. Erby Wilkinson, Major Professor

We have read this dissertation and recommend its acceptance:

Dave Slauson, Rick Woychik, Virginia Godfrey

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To the Graduate Council:

I am submitting herewith a dissertation written by Sharon Golda Witonsky entitled "Characterization of the Pathophysiology of Wiskott-Aldrich Transgenic Mice". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

CHARACTERIZATION OF THE PATHOPHYSIOLOGY OF WISKOTT-ALDRICH TRANSGENIC MICE

A Dissertation
presented for the
Doctor of Philosophy Degree
The University of Tennessee,
Knoxville

Sharon Golda Witonsky, DVM
December, 1997

Dedication

This thesis is dedicated to all of the people who have supported and encouraged me over the many years to pursue my dreams and goals.

Acknowledgements

I would like to thank all of my committee members, Dr. J. Erby Wilkinson, Dr. Dave Slauson, Dr. Rick Woychik and Dr. Virginia Godfrey, for their time and efforts over the last four years. I would like to thank my advisor, Dr. Erbby Wilkinson, for persuading me to come to Tennessee and for giving me the freedom and confidence to allow me to pursue my goals. I would like to thank all the members of the Wilkinson and Woychik labs for their support and efforts over the last four years, especially to Brad Yoder, Bill Richards, Ed Michaude, Carmen Foster, Nancy Neilsen, Marilyn Cottrell and Jane Czarra. A special appreciation goes to all of my friends and family who have helped me through the last several months in order to finish my thesis.

Abstract

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disease in which affected patients have any combination of the triad of classical symptoms of thrombocytopenia, eczema and immunodeficiencies. The gene has been cloned and sequenced, but its function is unknown. In order to determine the pathophysiology of the disease, transgenic animals have been generated which overexpress the normal Wiskott-Aldrich syndrome protein (WASP) in T lymphocytes. By characterizing the pathobiology and immunology of these mice, we will better understand the normal function of Wiskott Aldrich syndrome protein (WASP).

The WASP transgenic mice have a profound phenotype that is characterized most prominently by splenomegaly and lymphadenopathy. Peripheral blood leukocytes are mildly, but significantly reduced, with all lineages being affected. WASP transgenic mice also have a mild thrombocytosis and megakaryocytosis. Histologically, the animals have enlarged germinal centers and increased numbers of germinal centers and lymphoproliferation in lymph nodes and spleens. This is reflected by elevated *in vitro* proliferative responses by both B and T cells. Signal transduction experiments show increased phosphorylation of several proteins in WASP transgenic cells versus cells from non-transgenic littermates. Based on these findings, it is believed that overexpression of WASP protein either in an antigen dependent or independent manner upregulates the

immune response of B and T cells to various mitogens and pathogens. As a result, WASP transgenic mice develop chronic splenomegaly and lymphadenopathy due to the "activated" status of their immune cells. The differential phosphorylation of several proteins involved in signal transduction, even in the resting state, suggests that the immune systems of the WASP transgenic animals may be non-specifically activated. These same signal transduction proteins, which are affected by overexpression of the WASP protein are likely involved in the pathophysiology of WAS. Further efforts will be taken to identify these proteins and elucidate their role in both the pathophysiology of these transgenic mice as well as Wiskott-Aldrich syndrome. In the future, further experiments will be done to determine if this is an antigen dependent or independent response.

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LIST OF ABBREVIATIONS

AIHA – autoimmune hemolytic anemia

ABTS – 2,2'-azino bis 3-ethylbanzthiazoline-6-sulfonic acid

B220 – B cell marker

B71 – activation marker

B72 – activation marker

BME – 2-beta mercaptoethanol

BMTs – bone marrow transplants

BSA – bovine serum albumin

CD3 – T cell

CD4 – T cell, helper cell

CD8 – T cell, cytotoxic cell

CD28 – T cell, cell surface activation marker

CD40 – activation marker on antigen presenting cells

CD40L/gp39/CD154 – T cells, cell surface activation marker

CD43 – leukosialin, cell surface marker

CD44 – cell surface marker for memory cell

CD45RB – cell surface marker on T cells

cdc42hs – GTPase (GTP binding protein)

Con A – concanavalin A

CsCl -- cesium chloride

DN – double negative (CD4/CD8-)

DNA – deoxyribonucleic acid

DP – double positive (CD4+/CD8+)

EDTA – ethylene diamine tetratacetic acid

EBV – Epstein Barr Virus

EM – electron micrograph

ERM – ezrin-radixin-moesin

ERKs – extracellular signal regulated kinases

FACS – flow cytometry

FCS – fetal calf serum

fyn – src cytoplasmic tyrosine kinases

GBD – GTPase binding domain

GCN – guanidine thiocyanate

Grb-2 – adaptor protein

HRP – horse radish peroxidase

Ig – immunoglobulin

IL-2 – interleukin-2

IL-4 – interleukin-4

IL-6 –interleukin-6

Itk – non receptor protein tyrosine kinase

LPS – lipopolysaccharide

MAP – mitogen activated/associated protein

MBP – myelin basic protein

MCS – multiple cloning site

mel-14 – homing cell surface marker

nck – SH2/SH3 adaptor protein

p38 – stress activated MAP kinase

PAK – p21 activated kinase

PBS—phosphate buffered saline

PCR – polymerize chain reaction

PMNs – polymorphonuclear cells

rac—GTPases, GTP binding protein

rho – GTPase, GTP binding protein

RIDs – radioimmunodiffusion assay

RIPA – cell lysate buffer

RNA – ribonucleic acid

ROCK – Serine/threonine kinase (p160)

RT – reverse transcriptase

RT-PCR – reverse transcriptase

SHCs -- phosphoproteins

SP – single positive

TCA – trichloroacetic acid

TECs – thymic epithelial cells

TMB – substrate chromagen

TH1 – CD4+ helper cell, type 1

TH2 – CD4+ --type 2 helper cell

Tg – transgenic

WAS – Wiskott-Aldrich syndrome

wasp gene – Wiskott-Aldrich syndrome protein gene

WASP protein – Wiskott-Aldrich syndrome protein protein

PART I: GENERAL INTRODUCTION AND OVERVIEW

Chapter 1: Introduction

Wiskott-Aldrich syndrome (WAS) is one of the least understood, yet most intriguing, immunodeficiencies of humans. WAS is an X-linked recessive disease which classically involves thrombocytopenia, eczema, and B and T-cell immunodeficiencies (Cooper, 1968). The mean age of diagnosis based on clinical signs is <2 yrs., and many patients succumb to hemorrhage or septicemia, often due to secondary infections, early in their childhood (Sullivan, 1994). Patients who survive early childhood also have an increased incidence (>100X) of lymphomas, especially those associated with Epstein Barr Virus (EBV) with a mean age of onset of tumors of 9 yrs (Peacocke; Sullivan 1994). Patients are also prone to develop autoimmune conditions, such as autoimmune hemolytic anemia (AIHA), vasculitis, renal disease and arthritis (Sullivan, 1994).

Despite the hallmark signs, a definitive diagnosis of WAS has often been difficult because of the variance in both clinical signs and severity of the disease. The dermatitis may be transient, mild and/or absent and the histopathology of the dermatitis varies from atopic to seborrheic to exfoliative. The scalp, face, and buttocks are sites of predilection for WAS-related eczema. The severity of the thrombocytopenia in WAS patients is also somewhat variable. In some cases, it may be the only sign, and the diagnosis of WAS may be serendipitous, whereas in other cases the thrombocytopenia may be extremely severe and even fatal (Cooper, 1968).

Immune function in WAS patients ranges from asymptomatic to severely immunocompromised ; severely affected patients may die from bacterial or fungal infections. The most common immunological defects in WAS patients include mild lymphopenia, low levels of circulating IgM, increased levels of serum IgG and IgA, failure to produce antibodies in response to polysaccharide antigens, and a progressive decline in T cell functions. Some patients develop Coomb's positive anemia (O'Donnell, 1996).

The standard treatments for WAS patients include supportive care, splenectomies and/or bone marrow transplants (BMT), Interestingly, the older literature suggests that in some cases thymic transplants alone have been successful in some cases. Patients who receive BMTs still have an increased incidence of lymphomas, and patients who are only treated with supportive care, antibiotics and transfusions, often succumb to hemorrhage or septicemia before adulthood (Sullivan, 1994).

Clinically, some of the variability in symptoms can now be explained by the nature of the mutation within the *wasp* gene. The gene was originally cloned and sequenced by Derry et al (1994). It had previously been mapped to the region of Xp11.22-11.23 through the use of linkage analysis and mapping. Once the gene was identified and sequenced considerable work has been done to determine correlations between various genetic defects and severity of the clinical signs. Patients with mutations causing a frameshift or a mutation involving the putative GTPase binding domain (GBD) often

have the classical WAS syndrome, whereas patients with missense mutations may present with only one of the clinical findings, such as thrombocytopenia (Feathertree, 1997; Schindelhauer, 1996; Symons, 1996; Wengler 1995). Cases have also been noted in which patients with the same genetic defect, even within the same family, have different clinical presentations. This not only complicates the study of the pathophysiology of WAS, but it also suggests that significant modifier genes exist. Clearly, knowing the function and regulation of the *wasp* gene and its encoded protein would not only enhance our understanding of this syndrome but also allow us to potentially improve patient management through the use of pharmacological and gene therapies.

Mutations are very informative in the analysis of the function of proteins. In order to surmise the role of a protein, one can examine the phenotype of the patients in which the gene and its encoded protein have been altered. In particular, one can examine the histopathological changes seen in patients, as well as the results from immunological assays in order to identify what changes the dysfunctional protein causes. Because of the range in severity and nature of clinical signs, and the variety of genetic defects in the *wasp* gene and its encoded protein, there is a broad spectrum in the histopathological changes reported, but generally the lymphoid tissues, predominantly spleen and lymph nodes, are most consistently affected. Changes include a severe lymphoreticular cell hyperplasia with depletion of the T-cell areas. Thus the paracortex of the lymph nodes

and the periarteriolar sheaths of the spleen are depleted of small lymphocytes and expanded and replaced by large numbers of reticuloendothelial cells. In some cases, the normal architecture is effaced by these hyperplastic cells. Medullary cords in lymph nodes often contain abundant plasma cells. The thymus is normal or depleted (Cooper, 1968).

Affected patients generally have a progressive decrease in the *in vitro* proliferation of the T cells in response to mitogens. However, the basis for this depression in T-cell responsiveness is unclear. Other data suggests that the T and B cell lines from WAS patients have a restricted defect in their response to mitogens. Some reports correlate alterations in CD43 expression, glycosylation or function, with failure to respond to certain antigens (Lau,1992; Molina, 1993; O'Donnell, 1996; Siminovitch, 1993). Comparisons between different studies are difficult because of the marked differences in phenotype associated with different *wasp* mutations and differences in individual patients possessing the same mutation.

To begin to systematically address these questions, we generated transgenic mice which overexpress WASP under a T cell promoter. As with many gain of function experiments, we have identified a significant phenotype in these T cell specific WASP transgenic mice. We have determined that overexpression of WASP in T cells is associated with splenomegaly and lymphadenopathy characterized by germinal center hyperplasia. Based on immunologic assays, the cells appear "activated" and signal

transduction experiments show variances in phosphorylation status of several proteins. These data suggest that the WASP protein is involved in the immune response at the level of cell signaling. Overexpression of WASP protein in T cells is associated with changes in cell signaling which results in the activated status of both B and T cells. As a result of altered regulation, the mice develop germinal center hyperplasia, which is reflected as splenomegaly and lymphadenopathy. Future experiments will be conducted to determine whether this is an antigen dependent or independent process and to assess the consequences of this "activated dysregulated immune state" (i.e. does immune dysfunction lead to the development of autoimmune diseases and/or tumors?). More recently *in vitro* studies have associated WASP with the cytoskeleton and signaling molecules including actin molecules and cdc42. Under these conditions, changes in WASP results in changes in cell morphology. These *in vitro* cytologic changes may correlate with the *in vivo* changes seen in WAS patients including the characteristically small platelets and blunted microvilli on cells. Dysregulation of CD43, reported in some WAS patients is also associated with changes in cell morphology and immune function. Further studies need to be done to elucidate the association between these cytoskeletal proteins, including cdc42hs and actin, and WASP.

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PART II: PATHOLOGY OF WASP TG ANIMALS

Chapter 1: Abstract

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive condition for which the associated gene has recently been identified. The classical hallmark signs for WAS are thrombocytopenia, characterized by small platelets, eczema and a progressive B cell and T cell immunodeficiency. Affected patients have splenomegaly and lymphadenopathy which are characterized histologically by lymphorecticular cell hyperplasia. Here we have generated WASP transgenic animals whose expression of the transgene is limited to lymphoid tissues. These animals have a marked incidence of splenomegaly and lymphadenopathy, characterized by germinal center hyperplasia. Peripherally, they also have depressed white blood counts, elevated platelet counts, as well as megakaryocytosis in the bone marrow and spleen.

Chapter 2: Introduction

Wiskott-Aldrich syndrome (WAS) was originally characterized by Dr. Wiskott in 1932 based on 3 patients with congenital idiopathic thrombocytopenia, persistent infections, and bloody diarrhea. Classically, a diagnosis of WAS has been based on a triad of clinical signs including thrombocytopenia, eczema, and progressive B and T cell immunodeficiency. The mean age of diagnosis is <2yrs., and affected patients often succumb to hemorrhage or septicemia, often due to secondary infections (Sullivan, 1994)). Patients who survive are prone to lymphomas as well as autoimmune conditions, such as autoimmune hemolytic anemia (AIHA), vasculitis, renal disease and arthritis. More recently the gene has been mapped (Xp11.22-p11.23), cloned and sequenced (Derry, 1994). Once the gene was identified and cloned, it has become apparent that many patients with a defect in the *wasp* gene may not have all 3 clinical maladies. Many studies have been done to correlate the nature and location of the defect within the *wasp* gene and the severity of clinical disease. In many cases, correlations can be made between the defect (i.e. frameshift or mutation of the GTPase binding domain (GBD) and severity of clinical signs (i.e. all three syndromes vs. thrombocytopenia).

However, there remains considerable variability between the genetic defects and the clinical presentations in that other patients with the same exact genetic defect, even

within the same family, have a different form and severity of the disease. Some patients have normal serology, where as others have decreased IgM, and IgG; some have defects in cell proliferation assays; others may have defects in functional assays; some patients have alterations in cell surface marker expression, (i.e. CD43), which may affect cell function. In order to characterize the nature of this disease and the function of the protein under more deliberate/controlled conditions, WASP transgenic animals were generated. These animals will be and have been used to further elucidate the roles of this protein and its function in the pathophysiology of WAS.

Chapter 3: Materials and Methods

Genetics of WAS: Dr. Ute Francke's laboratory recently utilized positional cloning techniques to isolate a gene from Xp11.22-p11.23 that is mutated in WAS patients. The gene codes for a 510 amino acid proline rich protein that is expressed specifically in the lymphoid tissues and cell lines of lymphocytic and megakaryocytic origin.

Generation of construct and transgenic mice: The construct (Figure 2-1) was made in Dr. Richard Woychik's laboratory. It consists of the full length mouse *wasp* cDNA, which was obtained from Dr. Uta Francke's laboratory, and the murine V_B promoter, which was obtained as a gift from Dr. Uli Siebenlist and has been used to make over 30 transgenic lines (unpublished data). Standard methods were used for the generation, identification and maintenance of several lines of transgenic mice.

Genotype analysis: Genomic DNA was obtained by clipping approximately 2cm of the tail from mice at the time of weaning. The tails were digested overnight at 42^0C in 1 ml of proteinase K (1mg/ml) solution. Phenol was added and the DNA was extracted and precipitated with 2.5x's excess 95% ethanol. The DNA was resuspended in 400ul TE buffer.

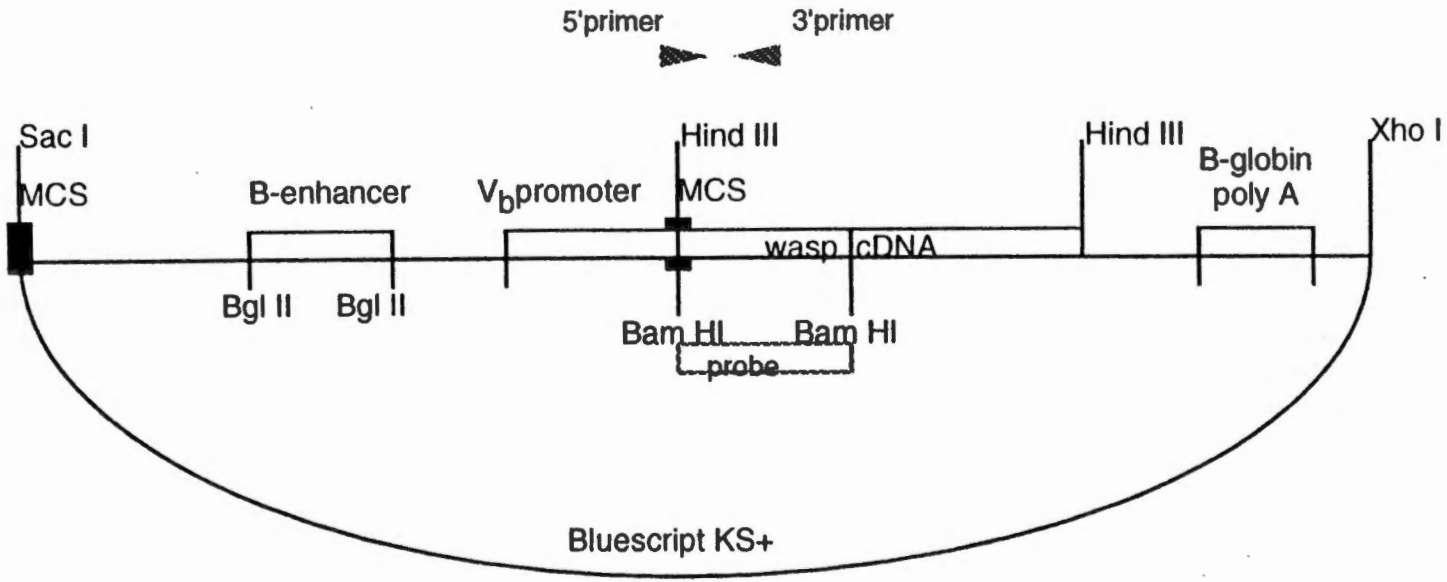


Figure 2-1: Map of construct for WASP transgenic mice, including probes and pcr primers used for genotyping.

Animals were genotyped by either Southern Blot analysis or PCR. For Southern blot analysis, tail DNA was digested with BAM and separated on a 0.8% agarose gel. Gels were denatured, neutralized and transferred onto Gene Screen using standard procedures. The probe, which was specific for the transgene, was an 850bp BAM fragment from the 5'end of the of the cDNA, including approximately 20bp of the multiple cloning site (MCS) of the construct. High stringency conditions were used (65°C, 0.2XSSC + 0.2%SDS, 0.5hrs). Transgenic animals have a band approximately 850bp.

For PCR analysis, DNA was generated as described above. Primers used were specific for the transgene. The 5' primer is a 20mer from the MCS of the construct (dCCC GGG CTG CAG GAA TTC GA). The 3' primer is a 21mer approximately 150 bp from the 5' end of the cDNA (dAAC TGT GGT TAG GCC AGT GTC C). PCR conditions were as follows (4.5 ul 10mM MgCL2, 5ul 10X buffer, 0.5ul 10U/ul Taq, 2ul 10mM dNTPs,100nm of each primer, 36.75ul ddH_20, 1ul tail DNA. Conditions for amplifying DNA were as follows: 94^{0}C 5min, 25 cycles (93^{0}C 45"", 62^{0}C 30"", 72^{0}C 30") 72^{0}C 5min. Products were run out on a 3% agarose gel. Presence of the transgene was revealed as an approximately 200bp fragment.

Maintenance of animals: Animals are maintained on an inbred FVB stock by mating heterozygous transgenic animals x non-transgenic FVB's. All mice were housed in a conventional environment, and fed a standard laboratory diet and water *ad libitum*.

Expression studies: Initially, RNA from spleens was analyzed, and then expression analysis of multiple tissues from each line was performed. Tissues collected included thymus, spleen, lymph node, brain, kidney, liver and intestines. Tissues were homogenized in 5M guaninidine thiocyantate (GCN), and then they were put on a cesium chloride (CsCl) cushion. Samples were spun overnight at 16K and then RNA pellets were resuspended, precipitated out and resuspended. (Maniatis) RNA concentrations were determined and aliquots were taken for RT-PCR. Samples were DNase treated [37^{0}C x 0.5hrs (RNA in total volume 20ul, RNasin 1ul of 10U/ul, RQ1DNase 2ul of 1U/ul, 115.75 DEPC H_2O, 11.25 DNase buffer mix) then phenol chloroform extracted, precipitated with 2.5X volume 95% ethanol and 0.3M NaOAc, and resuspended in depc H_20]. Reverse transcription was then performed on samples. RNA samples were heated in boiling water for 5min, then 34.4ul of reaction mix (8ul 10X RT buffer, 8ul 10mM dNTPs, 2ul 40U/ul RNasin, 8.4ul 46.7pM/ul random hexamer, 18.6ul dH_2O, 25ul RNA sample---per reaction) was added. Samples were divided and 1ul MMLV-RT enzyme was added to "+" samples. Samples were incubated at 23^{0}C for 10min, then 42^{0}C 45', then boiling water for 6min. Samples were precipitated out with NaCl and 95% EtOH, and resuspended in DEPC H_2O. PCR was performed on products as described above.

Preparation of Samples for *In Vitro* Assays: Lymphoid organs were minced using a 3ml syringe plunger and cells were put through 40u sieves. Red blood cells were lysed using an NH4-Tris HCl (0.16M NH4HCl, 0.17M Tris-HCl, pH7.2) solution. Cells were incubated in 2-5mls of lysis buffer for 2.5min. at 37C. Cells were washed and aliquots were made and stained with 1:10 dilution of 0.4% trypan blue (Sigma) to count viable cells. Dilutions for the assays were taken.

Blood Collection: Animals were anesthetized with Metafane following the UT approved lab animal protocol. Blood samples were obtained either by cardiac puncture with a 26g tuberculin needle or from the orbital sinus using a pasteur pipet. For white blood cell and platelet counts and differentials, samples were placed in K-EDTA tubes and counts were made using standard methods. For serology, blood samples were spun and serum was collected and stored at -70°C.

Serology: Radioimmunodiffusion kits (RID) were obtained from The Binding Site. Serum samples were diluted and the appropriate volume was added to the plate. Standards were run concurrently. Plots were made from standards and concentrations of the samples were determined.

Histopathology: Necropsies were performed on transgenic and non-transgenic mice from different age groups (<3 months, 3-9 months and >9 months). Tissues were fixed in 10% neutral buffered formalin, and processed and embedded routinely. Approximately 5u sections were stained with hematoxylin and eosin.

Chapter 4: Results

Expression Studies: Figure 2-1 is a diagram of the construct used for the generation of the WASP transgenic animals. At this time, several (9) transgenic lines exist which overexpress the normal *wasp* gene under a T cell specific promoter. The lines have been screened to determine which underwent germline transmission and expression studies have been done by RT-PCR to determine the pattern and level of expression. Initially RNA from spleens was analyzed to determine which lines expressed the transgene and then multiple tissue analysis were performed. Figure 2-2A shows RT-PCR of spleens from several different lines. The first 2 samples are wildtype spleen and brain. Figure 2-2B shows RT-PCR from one representative transgenic line. Expression is limited to lymphoid organs: thymus, spleen and lymph node. Based on these studies, work has focused on the 2 highest expressing lines with the most profound phenotype (Tg5550 and Tg5562). All data reported is from these two lines unless otherwise stated.

Gross Morphology: WASP transgenic mice exhibit marked splenomegaly and lymphadenopathy. Table 2-1 is a table of the gross necropsy results comparing transgenic animals from different age groups with their non-transgenic littermate controls. Columns 1 and 2 are for thymii which are increased or decreased in size

Figure 2-2 RT-PCR Expression Studies.

A. RT-PCR for wasp transgene from spleens of wild-type and transgenic animals of several different lines. A 100bp ladder has been used. The "+" RT reaction is followed by the "-" RT reaction. The first two samples are +/+ spleen and +/+ brain. Other samples included are from transgenic lines Tg5737, Tg5550 and Tg5562. The product is an approximately 200bp product.

B. RT-PCR of multiple organs from wild-type and transgenic animals. A 100bp ladder is present. The top row is the wild type samples, and the bottom row contains samples from transgenic animals. All samples are run with the "+" RT reaction followed by the "-" RT reaction. Samples include T-thymus, S-spleen, N-lymph node, I-intestine, L-liver, and K-kidney.

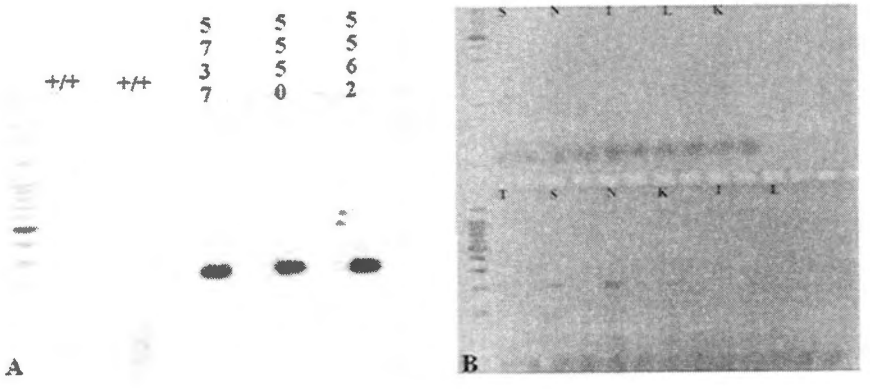

+/+
+/+
5 5 5
7 5 5
3 5 6
7 0 2
A
S N T L K
T S N K T L
B

Table 2-1: Gross Necropsy results from WASP transgenic animals and nontransgenic animals.
Animals are divided by transgenics and nontransgenic littermate controls. Animals are grouped as immature (<3months), adults (3-9months) and aged (>9months). Changes were determined as being >25% difference as determined grossly.

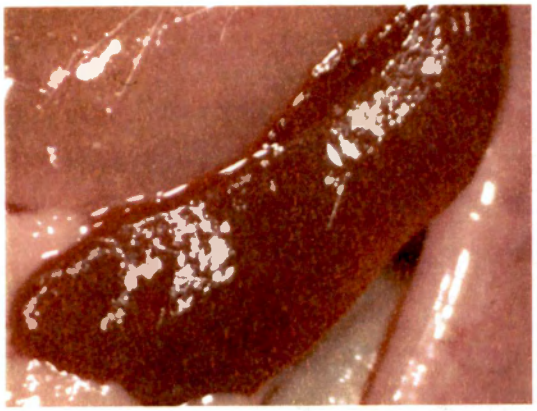
Age of Animals	inc. thymus	dec. thymus	splenomegaly	lymphadenopathy	hepatopathy	cachexia/mass
Transgenics						
<3 months	3/19	7/19#	18/19*	17/19*	0/19	0/19
3-9 months	3/18	5/18#	17/18*	17/18*	1/18	0/18
>9months	1/27	7/27	25/27*	25/27*	1/27	1/27
non-Tg animals						
<3 months	0/4	0/4	0/4	0/4	0/4	0/4
3-9 months	0/19	0/19	6/19	6/19	1/19	0/19
>9 months	0/16	2/16	2/16	2/16	0/16	0/16
				# $p < 0.05$		
				* $p<0.0001$		

grossly by at least 25% compared to normal. Column 1 is increased thymus size and column 2 is for decreased thymus size. Column 3 is splenomegaly which is >25% normal size as determined grossly. Column 4 is lymphadenopathy which can be asymmetric or symmetric changes in size grossly. Hepatopathy refers to any foci noted or increases in size that are >25% normal. Cachexia/wasting refers to lack of body fat, muscle wasting and thin appearance. The p value is <0.05 for thymic atrophy in the transgenics. Most significant is the incidence of splenomegaly and lymphadenopathy; Figures 2-3 and 2-4 illustrate the spleen and lymph node from WASP Tg mice at necropsy. Typically, the splenomegaly is uniform in appearance. There are often diffuse, multiple white <1mm foci apparent within the spleen. These represent areas of the germinal center hyperplasia. The lymphadenopathy is routinely bilateral and normally involves multiple lymph nodes including inguinal, axillary, superficial cervical and sometimes renal and sublumbar lymph nodes. Inguinal and axillary lymph nodes are often 3-5mm diameter.

Histopathology: Thymuses appear normal in the younger animals. There is a normal distribution of cells in both the cortex and medulla. Spleens and lymph nodes both show marked increases in the number of germinal centers present. Figure 2-5A is a 4X magnification of a typical histologic section of spleen illustrating the numerous germinal centers present. Figure 2-5B are higher magnifications of germinal centers which demonstrate the expansion of B cell populations, with many pyknotic nuclei and

Figure 2-3: Picture of a typical spleen from an adult WASP transgenic mouse (>6months old).

Figure 2-4: Picture of a typical lymph node from an adult WASP transgenic mouse (>6months old).



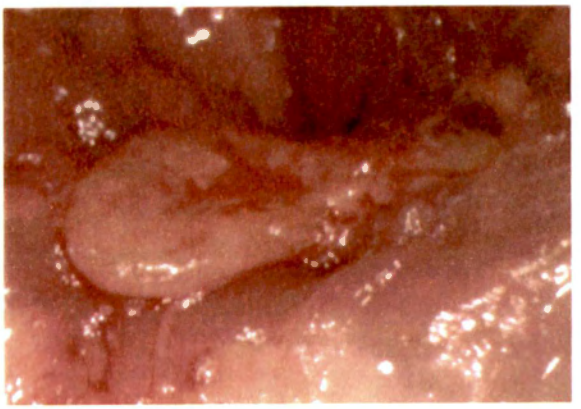
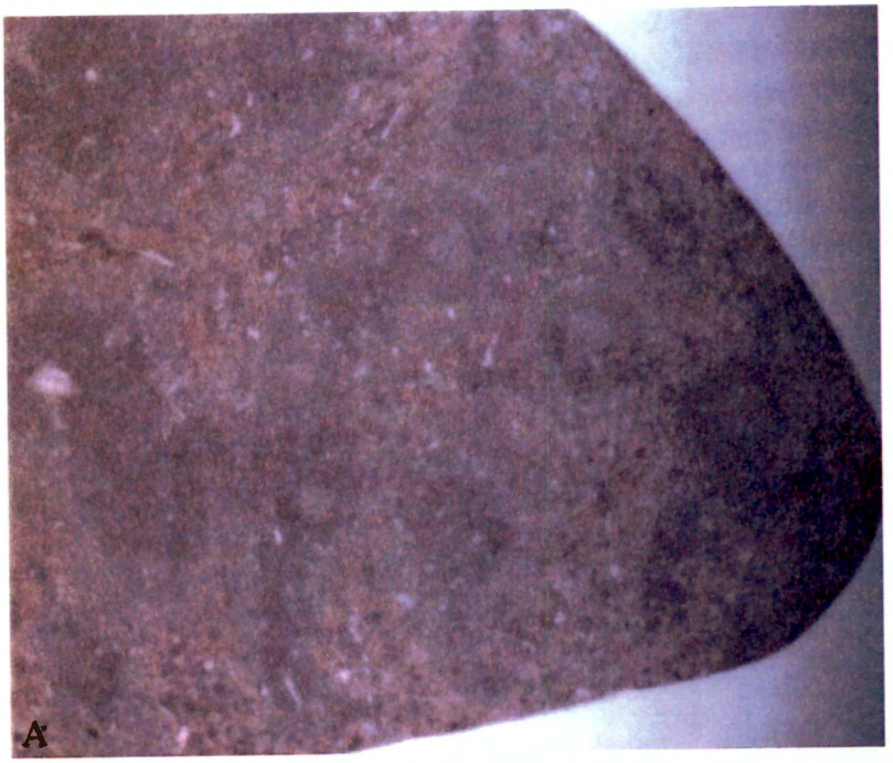
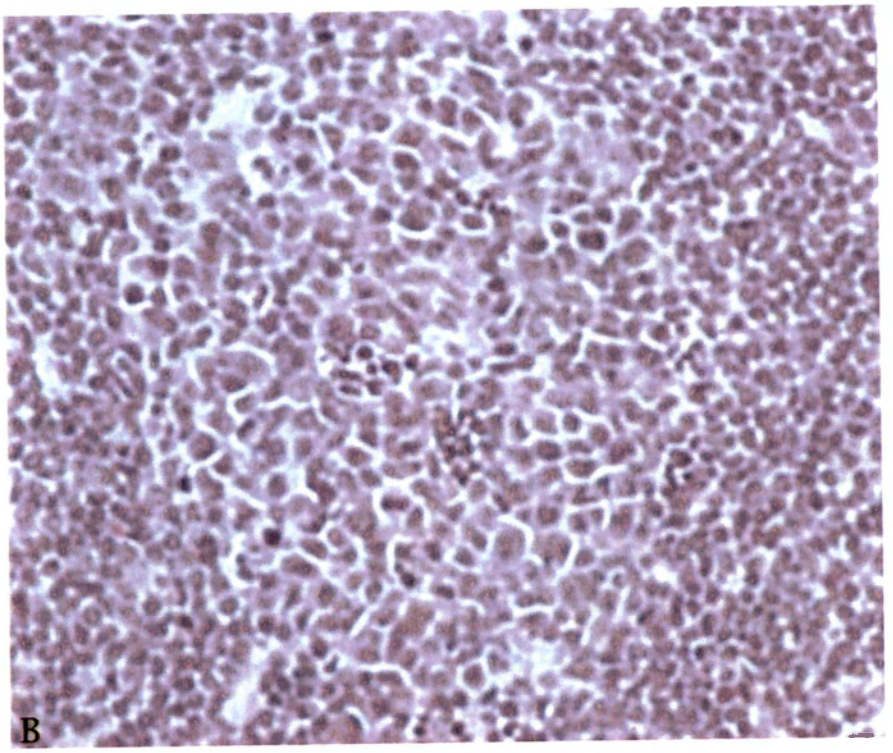


Figure 2-5: Histologic sections of a transgenic spleen.

A. Histologic section of transgenic spleen depicting multiple germinal centers (4x, hematoxylin and eosin).

B. Higher magnification of one germinal center with multiple apoptotic figures (20X, hematoxylin and eosin).


A


B

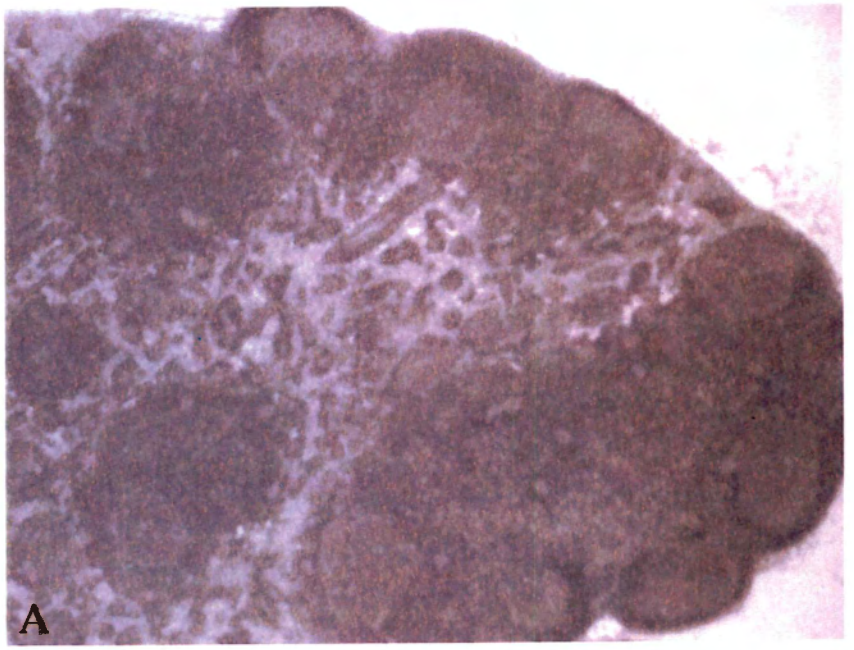
apoptotic cells present within the individual germinal centers. At higher magnifications, it can be seen that the endothelium of the venules are more cuboidal indicating that they are activated. Figure 2-6A is a 4X magnification of a typical lymph node section indicating the overall activated status of the immune system as evidenced by the appearance of numerous germinal centers. In general, the white pulp surrounding the blood vessels, i.e."the T cell areas" are remarkably normal. Only in some of the more severe cases, is there an expansion of the T cell areas. In addition, there is also marked medullary plasmacytosis evidenced in the lymph nodes (Figures 2-7A and 2-7B). In some lymph nodes there is a "disorganized" appearance to the medulla, which contains numerous plasma cells.

Based on the histopathology, it appears a strong primary immune reaction resulting from interactions between APC's, B cells and T cells is occurring in the transgenic animals. From these interactions some cells then form germinal centers; other cells undergo apoptosis. In some WASP Tg mice, the lymph nodes contain a few extremely large germinal centers and a paucity of cells in the medullary sinus and cords. Thisphase may represent a later stage in the normal immune response and may correlate with a different stage of the disease process. At higher magnifications, it can be seen that the endothelium of the venules, in the cortex and paracortex, are more cuboidal indicating that they are activated. Figure 2-6A and 2-6B are higher magnifications of the

Figure 2-6: Histologic section of a typical transgenic lymph node.

A. Histologic section of a typical transgenic lymph node with multiple germinal centers and a medullary plasmacytosis (4x, hematoxylin and eosin).

B. Higher magnification of one germinal center from the same lymph node (10x, hematoxylin and eosin).


A

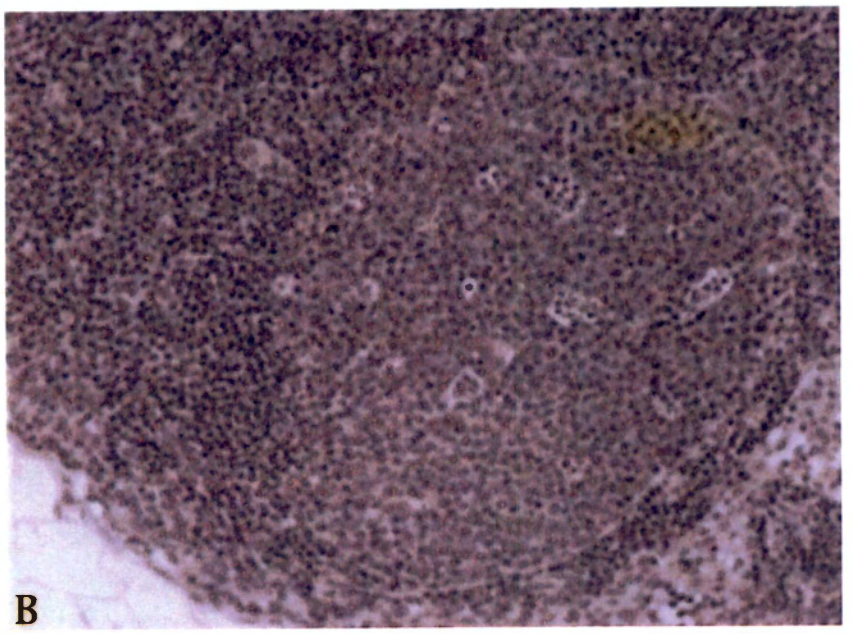

B

Figure 2-7: Medullary plasmacytosis in a transgenic lymph node.

A. Low magnification of the medulla of a transgenic lymph node illustrating hypercellularity (10x, hematoxylin and eosin).

B. Higher magnification (40x) of lymph node depicting medullary plasmacytosis (40x, hematoxylin and eosin).

B

A

lymph node illustrating the numbers of apoptotic cells and pyknotic nuclei present within the center.

There is a mild megakaryocytosis present in the spleens and bone marrows of WASP Tg mice. Figure 2-8A and B compare a section of spleen from transgenic versus wild type animals illustrating the increased number of megakaryocytes present in the spleen. Figure 2-9(A-C) show representative histologic sections of bone marrow from WASP-Tg and wild-type animals. The megakaryocyte to erythrocyte ratios from the bone marrow sections appear to be normal.

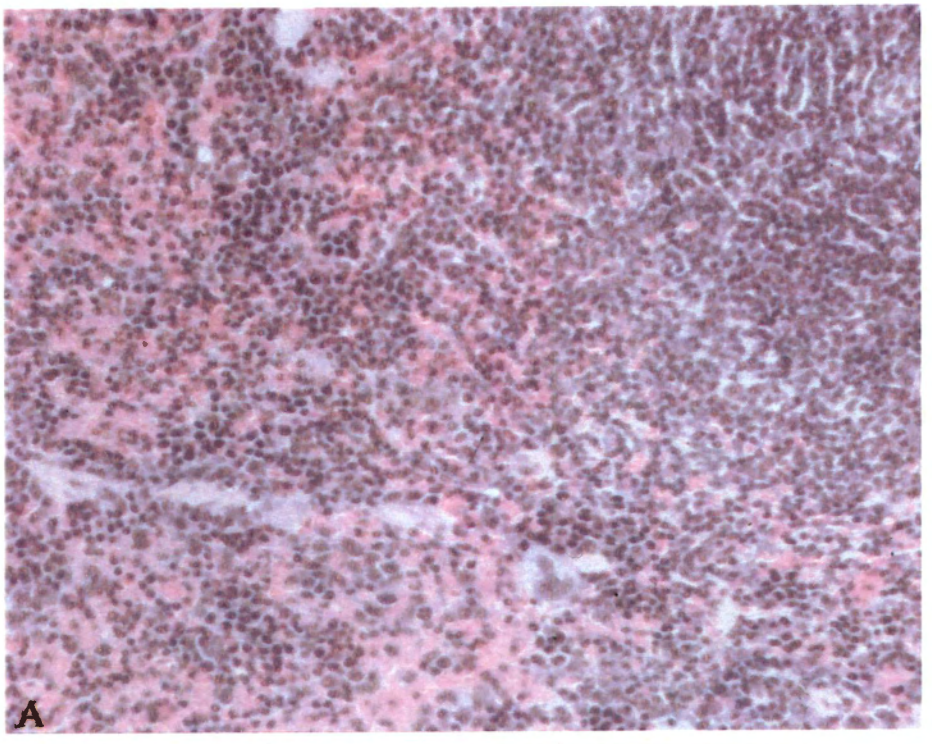
Hematology: In 3 experiments in which the white blood cell counts and differentials were performed, the transgenic animals have decreased total white blood cell counts compared to their non-transgenic littermate controls (Table 2-2). The leukocyte differential count illustrates that there is no particular population which is specifically affected; all lineages are decreased.

Platelet counts are mildly, but significantly ($p<0.03$) increased compared to non-transgenic animals. This correlates with the megakaryocytosis noted in spleens and lymph nodes histologically.

Figure 2-8: Comparison of megakaryocytes in spleens from wild-type and transgenic animals.

A. Histology of spleen from wild-type animal illustrating typical numbers of megakaryocytes (20x, hematoxylin and eosin).

B. Spleen from transgenic animal illustrating increased number of megakaryocytes (20x, hematoxylin and eosin).


A

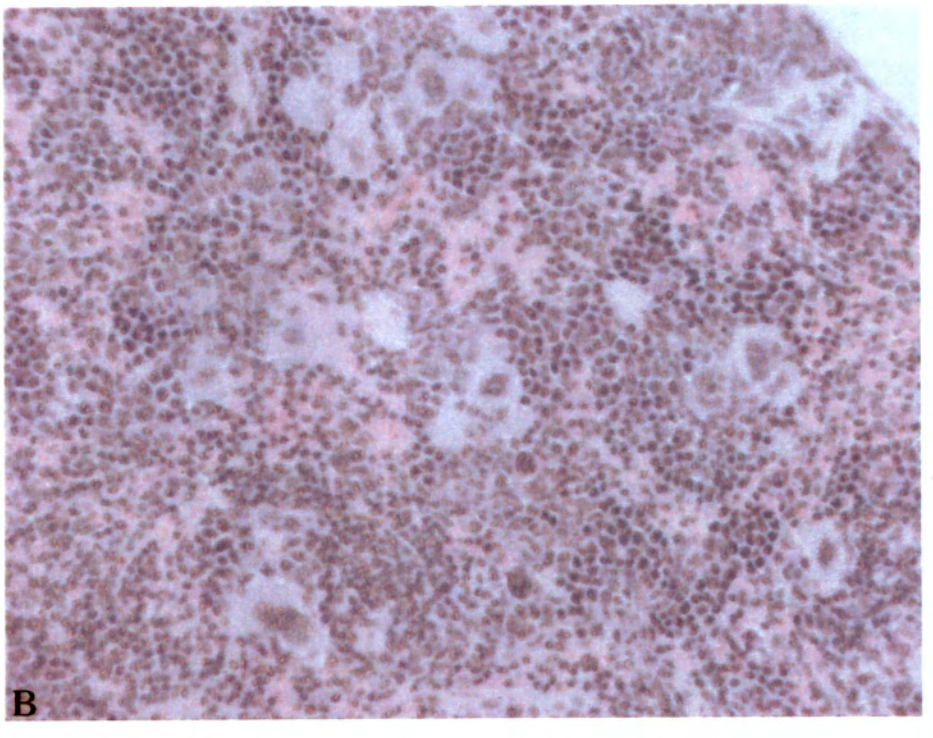
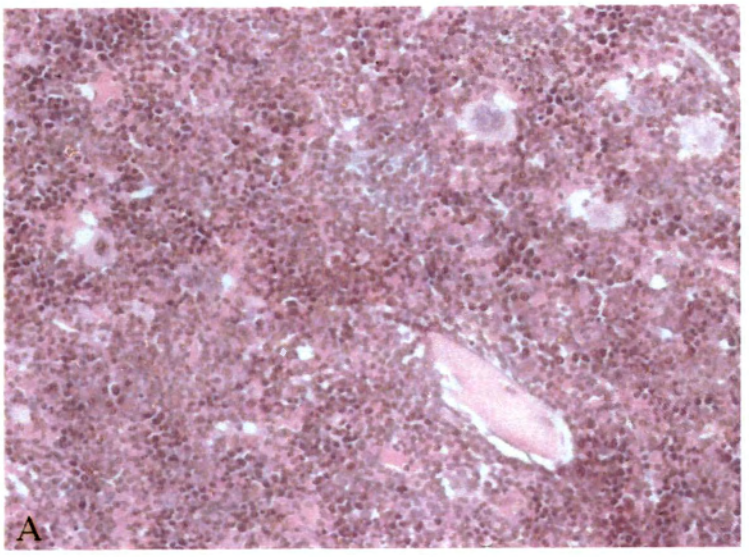

B

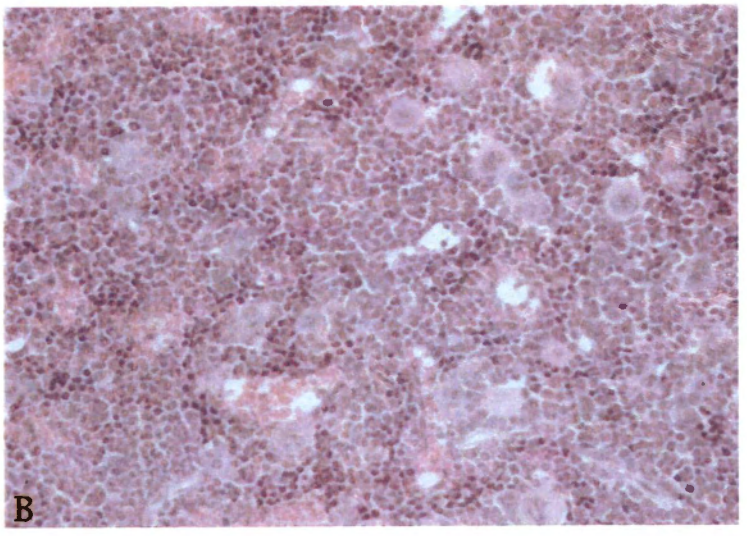
Figure 2-9: Histologic sections of bone marrow.

A. Low magnification of bone marrow from wild-type animal illustrating normal number of megakaryocytes (4x, hematoxylin and eosin).

B. Low magnification of bone marrow from transgenic animal illustrating increased number of megakaryocytes (4x, hematoxylin and eosin).

C. Higher magnification of same section of bone marrow from transgenic animal depicting relative megakaryocytosis (40x, hematoxylin and eosin).


A


B

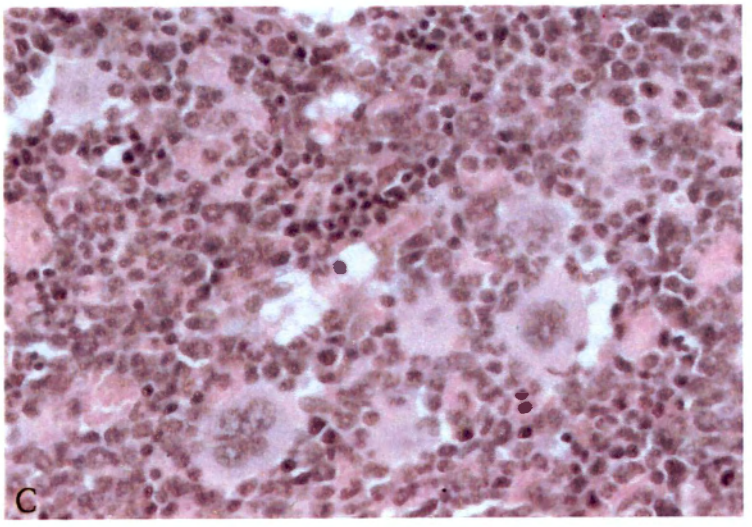

C

Table 2-2: White blood cell counts and differentials from wild-type and transgenic animals. Counts are in cells/ul.

	non-Transgenic (+/+)	Transgenic
	(n=9)	(n=13)
white blood cells (wbc)/ul	1814	848*
neutrophils	764	406*
lymphocytes	795	318#
monocytes	192	93#
eosinophils	6	2.1
basophils	53	30
platelets	1019	1215#
		* p<0.003
		# p<0.03

Serology: Table 2-3 reflects immunoglobulin levels from wildtype and transgenic animals which were analyzed as two batched samples. Transgenic animals have mild increases in IgG and IgA.

Table 2-3: Serology from wild-type and transgenic animals run as two batched groups. Immunoglobulin levels are reported as mg/dl.

Animals	IgA (mg/dl)	IgM (mg/dl)	IgG (mg/dl)
Transgenic (n=6)	2824.5	42.82	1111.42
Wild-type (n=4)	2407.8	30.42	1002.9

Animals	IgA (mg/dl)	IgM (mg/dl)	IgG (mg/dl)
Transgenic (n=12)	2372	108	3121
Wild-type (n=8)	1970	113	2906

Chapter 5: Discussion

Several transgenic lines have been generated using a T cell promoter and the mouse *wasp* cDNA. These 9 lines all underwent germline transmission (Witonsky, unpublished data) as determined by Southern blot analysis. Expression studies were done on spleens by RT-PCR to determine expression of the transgene in these lines and then multiple organ screens were done by RT-PCR to identify the pattern and level of expression of the transgene.

From Figure 2-2A it was determined that lines Tg5550 and Tg5562 had high levels of expression and this correlated with the severity of the phenotype. Lower expressing lines had a less severe phenotype grossly and histologically (Witonsky, unpublished data). Multiple tissue analysis was performed on all lines. This confirmed that, using routine RT-PCR conditions, expression was limited to lymphoid organs: thymus, spleen and lymph node. Based on high levels and pattern of expression and severity of phenotype, 2 lines (Tg5550 and Tg5562) were chosen to use for future experiments. Unless stated otherwise, all results are from animals from these two lines, Tg5550 and Tg5562.

Grossly, older WASP Tg animals have thymic atrophy. Histologically, the thymuses from younger animals appear to have normal distribution and numbers of cells in the cortex and medulla suggesting normal populations of immature cells and no obvious

defects in the maturation process. In the future, flow cytometry experiments will be done to determine if normal distributions and populations of cells are present. Blastogenesis and cytokine experiments will be done to determine if the thymocytes are functioning adequately compared to the non-transgenic littermates. Because thymuses appear grossly normal and histologically normal in the younger transgenic mice it is possible that the overexpression of the transgene does not affect thymic maturation or selection. Instead, thymic atrophy in the older animals may be a consequence of stress, disease or a secondary effect of the transgene. The apparent "activated" status of the spleens and lymph nodes, as described, may result in increased demand for cells to migrate from the thymus.

WASP transgenic animals develop splenomegaly and lymphadenopathy which is evidenced histologically by a marked increase in follicular germinal centers. Germinal centers reflect the activated status of the immune system, specifically B cell activation, which can occur through either T cell dependent or T cell independent pathways. Since the transgene is expressed from under a T cell promoter, it is presumed that the reaction in the transgenic animals is T cell dependent. This will be confirmed in future experiments. *In vivo* experiments using T cell independent (LPS) and T cell dependent (rabbit Ig) antigens. LPS will act as an antigen to stimulate a B cell response which can be measured using an ELISA which will detect IgG levels to LPS. (Foster, unpublished data). In addition, the activation of the immune system may be antigen independent, in

that overexpression of the WASP protein could directly causes increased T cell signaling.These activated T cells would then cause a T cell dependent B cell activation which would result in germinal center development. Alternatively, overexpression of the transgene may result in immune dysregulation such that the animals are immunocompromised or overstimulated (i.e. as in autoimmune disease). Thus, as a compensatory mechanism, the immune system continues to develop germinal centers, either because the host's immune system is not competent and/or functioning adequately in generating B cell responses. WASP Tg mice may or may not be able to produce protective immune responses. These animals have only mildly increased IgG and comparable IgA and IgM levels compared to their non-transgenic littermate controls despite their marked increases in germinal centers. (Table 2-3)

Another explanation for the B cell hyperactivity is that the T cell promoter is leaky and there is some small percentage of B cells that also express the transgene. As a result, the B cells are activated in an antigen independent manner, i.e due to overexpression of the transgene. Initial RT-PCR on separated populations of B cells and T cells demonstrate much higher levels of expression in the T cell population vs B cells, when comparable amounts of RNA were used. It is not clear whether the low amount of expression in the B cell samples is due to the contamination of T cells in the B cell sample or whether the T cell promoter is leaky, and there is a very low level of expression in the B cells (Witonsky, unpublished data). The more sensitive RNase

protection experiments will provide more quantitative information about the levels of expression of the transgene and endogenous levels in these cell populations because this will allow quantitation of expression based on correlating expression of the transgene and endogenous gene with 32P incorporation.

White blood cell counts and differentials demonstrate that the transgenic animals have significantly decreased WBC's compared to non-transgenic littermate controls. This depression is a pancytopenia, in that all populations are affected. Bone marrow analysis indicates that normal populations of precursor cells are present and the myeloid: erythroid ratio appears normal, suggesting that the defect is peripheral. If the lymphoid system is "activated" perhaps the remaining leukocyte populations are less stimulated. In contrast, if the overall lymphoid system is compromised, cytokines, chemokines, prostaglandins and co-factors which normally stimulate other immune cells (i.e. granulocytes and monocytes) may not be synthesized and/or released from the bone marrow and spleen. Finally, it is possible that there is just increased margination of cell populations to blood vessels and lymphoid organs (i.e. reflected as the lymphadenopathy and splenomegaly). The *wasp* gene is normally expressed in all hematopoietic precursors (Mantuano, 1993; Sullivan, 1994). Therefore, a leaky promoter, could allow expression of the transgene in monocytes and PMNs, which might result in decreased peripheral counts of these cells. These possibile explanations for the abnormal leukograms in

WASP Tg mice will be addressed in future RNase protection assays and thymic transplant experiments.

Despite having decreased WBC counts the transgenic animals have elevated platelet counts and a mild megakaryocytosis in the spleens and bone marrow. Possible causes of this are decreased consumption of platelets or increased production, perphaps secondary to a primary inflammatory condition. It is possible that the promoter is leaky and the transgene is expressed in platelet precursors causing increased production of platelets. This will be addressed in further RNase protection experiments. Depressed platelet function may also cause platelet numbers to be inreased due to increased demand or decreased consumption. Platelet function will be addressed by doing bleeding times on the mice (Subramaniam, 1996; Swank, 1996). Alternatively, elevated platelet counts may be due to increased (specific or nonspecific) inflammatory activity in the transgenic animals. In other species, inflammatory disease conditions, have been reported to increase platelet counts. (Sellon, 1997).

Overexpression of the transgene leads to lymphadenopathy and splenomegaly which is associated with a marked increase in numbers of germinal centers. Additionally, animals have mild megakaryocytosis and increased platelet counts but depressed white blood cell counts and differentials. Further experiments will be conducted to determine the expression of the transgene within specific hematopoietic cell populations, and to determine whether germinal center development is a direct or secondary consequence of

transgene expression, and to determine whether the B cell hyperplasia is antigen dependent or independent.

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PART III: IMMUNOPATHOLOGY OF WISKOTT-ALDRICH TRANSGENIC MICE

Chapter 1: Abstract

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disease in which affected patients may not appear to have any immunological disorders or they may have severe immunodeficiencies, autoimmune disorders and/or neoplastic conditions. Characterizing the immunological defects associated with WAS has been difficult both because of the range of clinical manifestations of the disease as well as the lack of understanding of the function of the WASP protein. In order to elucidate its role in WAS, transgenic animals were generated which overexpress the normal WASP protein. These animals develop splenomegaly and lymphadenopathy which is characterized by marked germinal center development. There is an increase in T and B cells in the spleens and lymph nodes as evidenced by flow cytometry, and these cells have an "activated phenotype". Both splenocytes and lymphocytes have increased CD45RB+ expression. B Lymphocytes have increased CD40+ expression, and increased numbers of B7-1+ and B7-2+ cells. T lymphocytes have upregulation of CD28+ expression. Both splenocytes and lymphocytes have increased numbers of CD43+ cells.

Chapter 2: Introduction

Patients affected with Wiskott-Aldrich syndrome (WAS) have a range of clinical signs depending on the nature and location of the mutation as well as other unknown factors. They may only have thrombocytopenia or they may have the classical triad of symptoms, including thrombocytopenia, eczema and progressive immunodeficiencies. The pathophysiology of the immunological disorders is not understood. Therefore, in order to dissect its role in the immune system, we have generated Wiskott-Aldrich transgenic mice which overexpress the normal WASP protein under a T cell promoter. As these mice age, they develop splenomegaly and lymphadenopathy. Histologically, both spleens and lymph nodes show marked increases in germinal center development, suggesting activation of the immune system. Overall, the *in vivo* and *in vitro* experiments support the activation of the immune system in the transgenic animals vs. their non-transgenic littermates. Immunoglobulin levels are elevated in transgenic animals. Flow cytometry demonstrates increased numbers of lymphocytes, both B and T cells in the spleens and lymph nodes. In addition, there appears to be an increased population of CD45RB+ cells in both spleens and lymph nodes. Lymphocytes from peripheral lymph nodes have an activated phenotype. There is an increase in CD40+ expression as well as upregulation of CD28. Lymph nodes contain an increased number

of B7-1+ and B7-2+ cells. It was also found that cell surface expression of the resting isotype of CD43(115kd) is increased on both splenocytes and lymphocytes and possibly decreased on thymocytes. *In vitro* blastogenesis results suggest that both T and B cells have increased proliferative indices when stimulated with T and B cell specific mitogens. In addition, production of IL-2 by stimulated spleen and lymph node samples is increased. Overall, the *in vivo* and *in vitro* experiments support the activation of the immune system in the transgenic animals vs. their non-transgenic littermates.

Chapter 3: Materials and Methods

Maintenance of animals and transgenic lines: Animals are on a congenic FVB stock, which is maintained by mating heterozygous transgenic animals x non-transgenic FVB's. All mice were housed in a conventional environment, and fed a standard laboratory diet and water *ad libitum*. Animals are genotyped by PCR using a 5' primer specific for the transgene, within the multiple cloning site of the promoter, and a 3' primer in the 5' end of the mouse *wasp* cDNA. This reaction yields an approximately 200bp product.

Blood Collection: Animals were anesthetized with Metafane (Pittman/Moore) following established procedures (UT protocol #A3668-01). Blood samples were obtained either by cardiac puncture with a 26g tuberculin needle or by collecting blood from the orbital sinus using a pasteur pipet. For white blood cell and platelet counts and differentials, samples were stored in K-EDTA tubes and counts were made using standard methods. For serology, blood was collected in serum tubes. Samples were spin and serum was collected and stored at -70°C.

Preparation of samples for *in vitro* assays: At necropsy, lymphoid organs were collected and put in 5ml aliquots of complete media (GIBCO RPMI 1640 supplemented with 5%

heat-inactivated fetal calf serum (FCS) Intergen , 2% antibiotic/antimycotic GIBCO (penicillin G/streptomycin, amphotericin B (2-Beta mercaptoethanol (BME) (3.4 x10-4volume/volume) and insulin/oxaloacetic acid (660mgoaa,40mg insulin,25mls 11mg/ml Na pyruvate/50mls water). Lymphoid organs were minced using a 3ml syringe plunger and cells were put through 40u sieves. Cells were washed and red blood cells were lysed using a NH4 Tris-HCl (0.16M NH4HCl, 0.17M Tris, pH7.2) solution. Samples were incubated in 2-5ml of solution for 2.5min. at 37C. Aliquots were made and stained with 1:10 dilution of trypan blue (0.4% GIBCO) to count viable cells. Dilutions for the assays were taken. Most of the mice used for in vitro assays were at least three months old, and most of the mice were greater than six months old. Most of the mice already had enlarged spleens and lymph nodes and many thymii had already undergone deletion at the time of euthanasia.

Serology: Radioimmunodiffusion kits (RID) were obtained from The Binding Site. Serum samples were diluted and the appropriate volume was added to the plate. Standards were run concurrently. Plots were made from standards, and concentrations of the samples were determined.

Blastogenesis Assays: Lymphocytes were plated in triplicate at 2 X 10^6/ml,100ul per well, in complete media with 5% FCS. Cells were stimulated for 48hrs with either

Concanavalin A (Con A 2.5ug/ml Sigma), anti-CD3monoclonal antibody (100ul of 1:1000 dilution of ascites purified anti-CD3) or lipopolysaccharide (LPS) (Sigma LPS 0111B4 10ug/ml). Wells were then pulsed with 1uCi/ well of ^{3}H and they were harvested after 18-24 hours. Results were calculated as stimulation indices, which is the ratio of ^{3}H incorporation in stimulated to non-stimulated cells.

Cytokine Analysis: Cells were plated at 2 x 10^{6}/ml in 1ml cultures in complete media. Cell cultures were stimulated with Con A at 2.5ug/ml. Supernatants were collected at 0 and 16-18hrs. Samples were frozen (-70^{0}C) until ELISAs were run to determine cytokine levels.

ELISAs for IL-2: Immulon^{R} plates were coated with 50ul/well of rat anti-mouse IL-2 antibody (2ug/ml in 0.1M $NaHCO_3$, pH8.2) (Pharmingen) overnight at 4^{0}C. Plates were washed with PBS/ Tween (0.05%) and then blocked with 200ul/well of PBS with 10% heat inactivated FCS for 2hrs at room temperature. Samples and standards were added at 100ul/well. Standards were diluted in blocking buffer. Samples were incubated at room temperature for 4 hrs or overnight at 4^{0}C or 2hrs at 37oC. Plates were washed and biotinylated rat anti-mouse IL-2 (Pharmingen) 1ug/ml in PBS + 10%FCS was added at 100ul/well and incubated at room temperature for 45 minutes. Plates were washed and avidin-peroxidase (100ul/well of a 1:400 dilution of 1mg/ml stock in PBS + 10% FCS)

was added. Plates were incubated for 30min. at room temperature and then washed. ABTS with H_2O_2 was added as the substrate and plates were read in 30min at 405nm.

IL-4 and IL-6 ELISAs (ENDOGEN): Plates were coated overnight with 100ul/well of diluted coating antibody (40ul stock/11mls assay buffer). Plates were blocked with 200ul/well of assay buffer (PBS +2%BSA, 0.01% thimerosal) at room temperature for 1hr. Plates were washed in wash buffer (1xPBS + 0.2% Tween) and diluted standards and samples were added 50ul/well plus 50ul/well of assay buffer. Samples were incubated overnight at room temperature. Plates were washed. Biotinylated antibody 100ul/well (35ul stock/11mls assay buffer) was added and plates were incubated for 1hr at room temperature. Plates were washed and 100ul/well of 1:4000 of HRP-streptavidin (Zymed) was added. Plates were incubated for 30min at room temperature and then washed. TMB substrate (DAKO) was added (100ul/well) and incubated for 30min at room temperature in the dark. Stop reaction (0.18M sulfuric acid) was added at 100ul/well and plates were read at 450nm.

Flow Cytometry: Cell suspensions were diluted to 1 x 10^6/ml. Cells were washed with ice cold wash buffer (1xPBS +1%BSA +0.2% Na azide). Pellets were stained with either fluorescin isothiocyanate (FITC- CD3)/ physoerythrin (PE) B220; FITC CD8/ PE CD4; biotinylated mel-14/PE CD44/ FITC-CD45RB, streptavidin C; FITC CD40/ PE gp39

(CD40L); biotinylated CD28/ FITC B7-1/PE B7-2, streptavidin C; FITC CD43. Stains were added at approximately 1ug/10^6 cells and incubated for 30min at 4^0C. Samples were washed and for biotinylated samples 1ug streptavidin cytochrome c was added and samples were incubated for an additional 30min at 4^0C. Cells were washed and resuspended in flow buffer. Flow samples were run on a Becton-Dickinson FACscan instrument. All monoclonal antibody reagants were from Pharmingen. Samples were analyzed using Cell Quest software (Macintosh).

Chapter 4: Results

All results were obtained from the two highest expressing WASP transgenic lines (Tg5550 and Tg5562) unless otherwise stated.

White blood cell counts and differentials: Transgenic animals have depressed white blood cell counts compared to their littermate controls (Table 3-1). All populations of leukocytes are affected equally, including lymphocytes, neutrophils, basophils, and monocytes. Counts are approximately 50% of the littermate controls. Platelet counts are elevated in the peripheral blood compared to non-transgenic littermate controls. Bone marrow analysis was done to examine precursor cells. By histological exam, the myeloid: erythroid ratio is normal and comparable to the non-transgenic littermate controls. However, histologically, there is a mild megakaryocytosis in the bone marrow and spleens.

Serology: On serum samples compiled from two different assays, transgenic animals have mildly elevated IgG levels. (Table 3-2) IgA levels and IgM levels are comparable for both transgenic and non-transgenic littermate controls.

Table 3-1: White blood cell counts and differentials from wild-type and transgenic animals. Counts are in cells/ul.

	non-Transgenic (+/+) (n=9)	Transgenic (n=13)
white blood cells (wbc)/ul	1814	848*
neutrophils	764	406*
lymphocytes	795	318#
monocytes	192	93#
eosinophils	6	2.1
basophils	53	30
platelets	1019	1215#
		* $p<0.003$
		# $p<0.03$

Table 3-2: Serology from wild-type and transgenic animals analyzed in two batched groups of samples. Levels are in mg/dl.

Animals	IgA (mg/dl)	IgM (mg/dl)	IgG (mg/dl)
Transgenic (n=6)	2824.5	42.82	1111.42
Wild-type (n=4)	2407.8	30.42	1002.9

Animals	IgA (mg/dl)	IgM (mg/dl)	IgG (mg/dl)
Transgenic (n=12)	2372	108	3121
Wild-type (n=8)	1970	113	2906

Blastogenesis: For thymus samples, proliferative responses were tested to Con A. Table 3-3 demonstrates that cells from transgenic animals had mildly elevated stimulation indices compared to their littermate controls. Transgenic cell samples produced mean standard indices of 86.86 vs. 76.26 for non-transgenic littermate controls ($p>0.1$).

Spleen samples were tested for their ability to respond to T cell mitogens, Con A and anti-CD3, as well as LPS, which was used as a B cell mitogen. Results from Table 3-3 show that all transgenic samples produced elevated proliferative responses compared to non-transgenic cell samples; p values were <0.075 for Con A anti-CD3 proliferative responses. Mean stimulation indices for Con A stimulated spleen samples from transgenic animals were 42.68 vs. 14.62 for littermate controls, and mean stimulation indices for anti-CD3 stimulated samples were 14.0 vs. 4.52 for controls.

Samples from lymph nodes were tested for both T and B cell mitogenic responses (Table 3-3). Transgenic animals produced hyperproliferative responses to Con A, anti-CD3 and LPS; p values for stimulation indices to Con A were <0.05. Transgenic animals produced mean standard indices of 9.88 versus controls were 4.52.

Cytokine Analysis: Only a few samples from the thymus have been analyzed. Samples from spleens and lymph nodes were analyzed for *in vitro* production of cytokines. ELISAs were run on supernatants from stimulated and non-stimulated cell cultures at 0 and 16-18hrs. Tables 3-4 to 3-6 show comparisons of cytokine production

Table 3-3: Blastogenesis results to different mitogens from thymus, spleen, and lymph node samples from transgenic and wild-type animals. For the transgenic samples n=6 and for the wild-type samples n=3. Transgenic samples are denoted as (T/+) and wild-type are labeled as (+/+).

	thymus T/+	thymus +/+	spleen T/+	spleen +/+	Lymph Node T/+	Lymph Node +/+
con A	86.86	76.26	42.68*	14.62	9.88#	4.52
CD3	N.D.	N.D.	14.02*	4.52	2.63	1.15
LPS	N.D.	N.D.	13.55	6.96	3.65	2.11

* p<0.075
\# p<0.05

Table 3-4: IL-2 production (pg/ml) by lymphoid cells from transgenic and wild-type animals. Transgenic samples are labeled as (T/+); wild-type samples are labeled as (+/+). For the transgenic samples n=6; for the wildtype samples n=3.

Composited Samples	thymus	thymus	spleen	spleen	Lymph Node	Lymph Node
	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)
Tg5562 12/96	0	0	652	599	588	0 (n=1)
5/21/97	N.D.	N.D.	6070	5657	5476	4941
7/21/97	426.3	766 (n=2)	5954	5416	5827	5477 (n=1)

Table 3-5: IL-4 production (pg/ml) by thymocytes, splenocytes and lymphocytes from transgenic and wild-type animals. Transgenic samples are marked as (T/+), and wild-type samples are labeled as (+/+). For the transgenic samples n=6; for the wildtype samples n=3.

Composited Samples	thymus T/+	thymus (+/+)	spleen T/+	spleen (+/+)	Lymph Node T/+	Lymph Node (+/+)
12/96	116	0 (n=1)	198	154	150	39 (n=1)
5/21/97	N.D.	N.D.	47	N.D.	20.5	23
7/21/97	8.57	40 (n=2)	31	29	25.5	N.D.

Table 3-6: IL-6 production (pg/ml) by transgenic and wild-type animals. Transgenic samples are labeled as (T/+), and wild-type samples are labeled as (+/+). For the transgenic samples n=6; for the wildtype samples n=3.

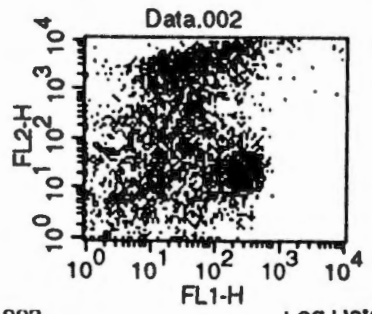
Composited Samples	thymus	thymus	spleen	spleen	Lymph Node	Lymph Node
	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)
12/96	33 (n=2)	42 (n=1)	198	N.D.	92	125 (n=1)
5/21/97	N.D.	N.D.	129	90.5 (n=1)	N.D.	N.D.
7/21/97	N.D.	N.D.	52	98 (n=3)	22	19 (n=1)

(pg/ml) by splenocytes and lymphocytes from transgenic and non-transgenic animals. Values are for stimulated samples at the 16-18hr time point. Non-stimulated and 0 hr samples are not reported here because values appear near baseline level. IL-2 levels are higher from both spleen and lymph node samples in transgenic vs. littermate controls (Table 3-4). IL-4 and IL-6 levels from transgenic samples are comparable to non-transgenic samples (Tables 3-5 and 3-6).

Flow Cytometry: Table 3-7 shows the flow cytometry data from thymus, spleen and lymph node samples. Both spleen (Figure 3-1, Appendix 3-1) and lymph node samples (Figure 3-2, Appendix 3-2) had increased percentages of T cells (FITC CD3) and B cells (PE B220). In the lymph nodes, there appear to be some double positive cells, but this is partly due to autofluorescene and not as discriminate gating as possible. Samples from lymph nodes (Figure 3-3, Appendix 3-3) had increases in CD40+ cells, suggesting B cells and/or APCs are activated. There are very few CD40L staining cells which is probably because most CD40L cells are CD4+, which means that only a small perceentage of the cells would be CD40L+ anyway. In addition, in unstimulated CD4+ cells, there is only a very small percentage of cells which are CD40L/gp39+ (Roy, 1993). Transgenic spleen samples had approximately the same number of CD40+ cells as nontransgenic littermate controls (Witonsky, data not shown). Cells from lymph nodes

Table 3-7: Summary of flow data analyzed for WASP transgenic animals. Results are reported relative to findings on wild-type animals.

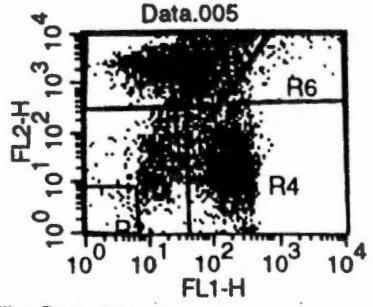
Stain	Thymus	Spleen	Lymph Node
CD3/B220	N.D.	Increased CD3	Increased CD3
		Increased B220	Increased B220
CD4/CD8	NSF	Increased CD8+ dull	4expts dec CD4+ dull + bright
			2expts. incr. CD4+ bright
CD40/CD40L(gp39)	ND	NSF	Increased CD40+
CD28/B71/B72	NSF	mild increase CD28	upregulation CD28+
		Increase B71+	increased B71+, B72+
		Increase B72+	increased B71+/B72+
mel-14/CD44/CD45R	ND	Increased CD45RB+ dull	Increased CD45RB+ dull and bright
CD43	Decrease CD43+ dull	Incr. CD43+ dull+bright	Incr. CD43+dull+bright



File: Data.002
Sample ID: 3/220
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 3758
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 32s1
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	3758	100.00	37.58
R2	221	5.88	2.21
R3	1213	32.28	12.13
R4	1177	31.32	11.77
R5	1336	35.55	13.36
R6	68	1.81	0.68

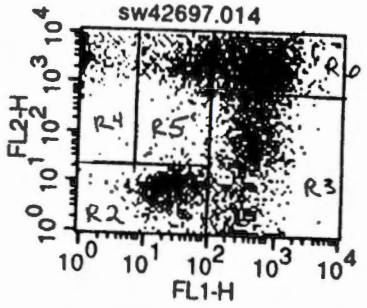


File: Data.005
Sample ID: 3/220
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 7451
X Parameter: FL1-H (Log)

Log Data Units: Line
Patient ID: 49s1
Case Number:
Panel:
Gate: G1
Total Events: 1000(
Y Parameter: FL2-H

Region	Events	% Gated	% Total
R1	7451	100.00	74.51
R2	33	0.44	0.33
R3	878	11.78	8.78
R4	3325	44.62	33.25
R5	3156	42.36	31.56
R6	122	1.64	1.22

Figure 3-1: Representative sample of results from wild-type (32) and transgenic (49) spleen cells stained for FITC-CD3 and PE-B220. Transgenic cells have increased numbers of CD3 (R4) and B220 (R5) cells compared to wild-type. Region 1(R1) = lymphocytes gated; R2 = unstained cells; R3 = unstained; R4 = CD3+; R5 = B220+; R6 = CD3+/B220+.

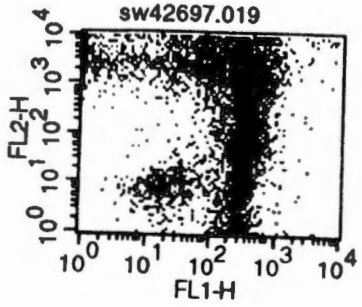


Region Statistics

File: sw42697.014
Sample ID: 3/220
Patient Name:
Tube:
Acquisition Date: 26-Apr-97
Gated Events: 5407
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 51In1
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	5407	100.00	54.07
R2	957	17.70	9.57
R3	1458	26.97	14.58
R4	175	3.24	1.75
R5	467	8.64	4.67
R6	2031	37.56	20.31



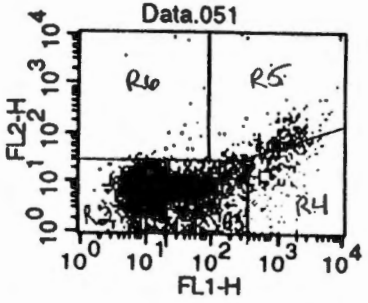
Region Statistics

File: sw42697.019
Sample ID: 3/220
Patient Name:
Tube:
Acquisition Date: 26-Apr-97
Gated Events: 8084
X Parameter: FL1-H (Log)

Log Data Units: L
Patient ID: 144In
Case Number:
Panel:
Gate: G1
Total Events: 10(
Y Parameter: FL2

Region	Events	% Gated	% Total
R1	8084	100.00	80.84
R2	447	5.53	4.47
R3	3893	48.16	38.93
R4	345	4.27	3.45
R5	643	7.95	6.43
R6	2098	25.95	20.98

Figure 3-2: Representative sample of results from wild-type (51) and transgenic (144) lymph node cells stained for CD3 and B220. Transgenic animals have increased numbers of CD3 cells. R1=lymphocytes; R2=unstained; R3=CD3+; R4=B220+; R5=B220+bright; R6=CD3+bright.

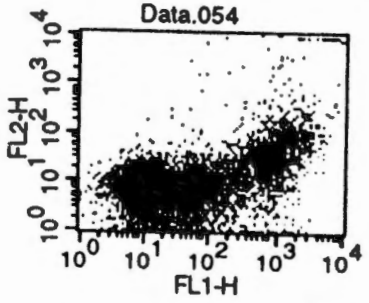


Region Statistics

File: Data.051
Sample ID: cd40/40L
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 5748
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 32ln4
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	5748	100.00	57.48
R2	3864	67.22	38.64
R3	1435	24.97	14.35
R4	252	4.38	2.52
R5	263	4.58	2.63
R6	20	0.35	0.20



Region Statistics

File: Data.054
Sample ID: cd40/40L
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 8049
X Parameter: FL1-H (Log)

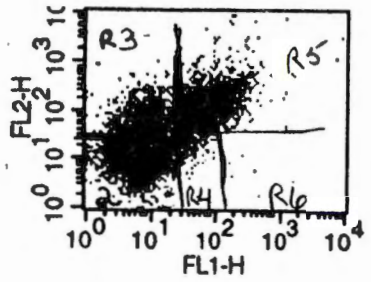
Log Data Units: Linear Values
Patient ID: 50ln4
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8049	100.00	80.49
R2	4127	51.27	41.27
R3	2412	29.97	24.12
R4	893	11.09	8.93
R5	698	8.67	6.98
R6	43	0.53	0.43

Figure 3-3: Flow data from lymph node of wild-type (32) and transgenic (50) cells stained for FITC-CD40 and PE-CD40L. Transgenic cells have an increase in CD40+ cells (R4 and R5). R1=lymphocytes; R2=unstained; R3=CD40+dull; R4=CD40+; R5=CD40+bright; R6=CD40L+.

had an upregulation (increased number of positive cells) for CD28+ on the cell surface (Figure 3-4, Appendix 3-4), and B cells had increased numbers of B7-1+, B7-2+ and double positive B7-1/B7-2 cells, suggesting that both T and B cells are activated. Gating on the triple stained populations was based on how the populations appeared as the cells were collected. It was easier to discern populations before the total 10,000 events were collected. Unstained cells appeared normal when collected and the amount of autofluorescence did not significantly affect the interpretation of these results. Splenocytes had mild increases in numbers of CD28+ cells, as well as increases in the number of B71+ and B72+ cells. (Figure 3-5, Appendix 3-5). Both splenocytes and lymphocytes had upregulated expression of CD45RB. For splenocytes (Figure 3-6, Appendix 3-6), it appears to be a decrease in CD45RB+ bright cells and an increase in CD45RB+ dull cells, whereas lymphocytes (Figure 3-7, Appendix 3-7) had an upregulation of CD45RB+ bright cells. There was both an increase in number of cells that were CD45RB+ bright as well as an increase in cell surface expression of CD45RB+ on the positive staining cells. No significant differences in CD44 or mel-14 were detected. Again, gating of these populations is based on the different populations which this author normally identifies. There was some autofluoresence present, but the unstained populations appear normal.

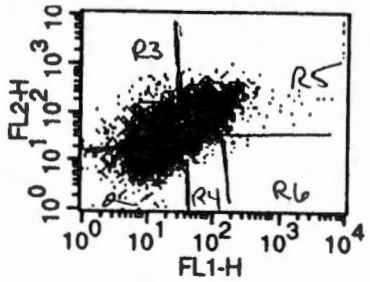
Finally, samples were also stained for CD43 expression. Thymocytes had a decreased number of CD43+ dull cells (Figure 3-8 and 3-9, Appendix 3-8). Spleen



File: Data.120
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8592
X Parameter: FL1-H (Log)

Log Data Units: Li
Patient ID: 134ln5
Case Number:
Panel:
Gate: G1
Total Events: 100
Y Parameter: FL2-

Region	Events	% Gated	% Total
R1	8592	100.00	85.92
R2	3582	41.69	35.82
R3	1716	19.97	17.16
R4	1367	15.91	13.67
R5	1829	21.29	18.29
R6	1	0.01	0.01

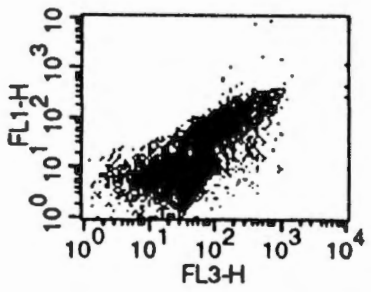


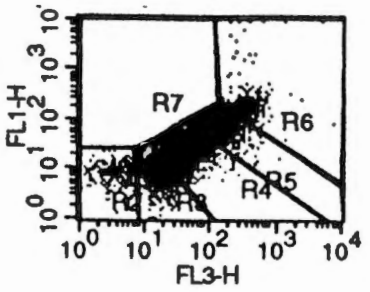
File: Data.125
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 9474
X Parameter: FL1-H (Log)

Log Data Units: L
Patient ID: 139ln
Case Number:
Panel:
Gate: G1
Total Events: 10(
Y Parameter: FL2

Region	Events	% Gated	% Total
R1	9474	100.00	94.74
R2	2205	23.27	22.05
R3	1248	13.17	12.48
R4	3540	37.37	35.40
R5	2333	24.63	23.33
R6	1	0.01	0.01

Figure 3-4: Representative sample of wild-type (134) and transgenic (139) lymph node cells stained for CD28/B71/B72.
Figure A depicts FITC-B71 vs. PE-B72. R1=lymphocytes; R2=unstained; R3=B72+; R4=B71+; R5=B71+/B72+; R6=B71+bright.





File: Data.120
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8592
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 134ln5
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL1-H (Log)

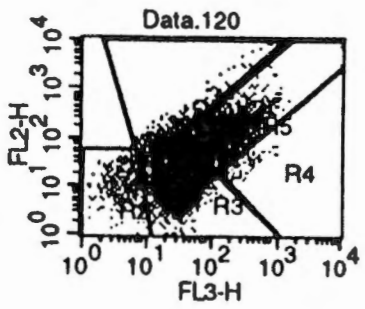
Region	Events	% Gated	% Total
R1	8592	100.00	85.92
R2	324	3.77	3.24
R3	2234	26.00	22.34
R4	3751	43.66	37.51
R5	1597	18.59	15.97
R6	543	6.32	5.43
R7	111	1.29	1.11

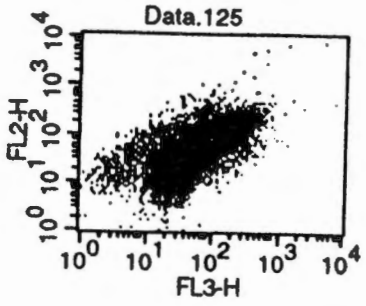
File: Data.125
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 9474
X Parameter: FL3-H (Log)

Log Data Units: Lin
Patient ID: 139ln5
Case Number:
Panel:
Gate: G1
Total Events: 1000
Y Parameter: FL1-H

Region	Events	% Gated	% Total
R1	9474	100.00	94.74
R2	294	3.10	2.94
R3	1603	16.92	16.03
R4	4631	48.88	46.31
R5	2267	23.93	22.67
R6	598	6.31	5.98
R7	105	1.11	1.05

Figure 3-4B depicts CD28 (FL3) vs. B71 (FL1). R1= lymphocytes; R2=unstained; R3=CD28+dull; R4=CD28+; R5= CD28+ bright; R6=CD28+/B71+; R7=B71+.





File: Data.120
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8592
X Parameter: FL3-H (Log)

Log Data Units: L
Patient ID: 134In
Case Number:
Panel:
Gate: G1
Total Events: 10(
Y Parameter: FL2

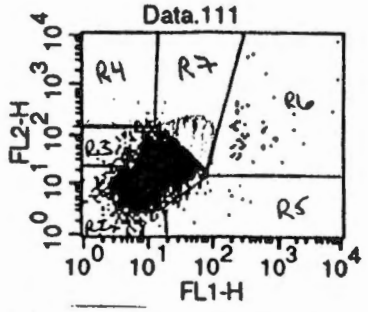
Region	Events	% Gated	% Total
R1	8592	100.00	85.92
R2	368	4.28	3.68
R3	4769	55.51	47.69
R4	112	1.30	1.12
R5	2850	33.17	28.50
R6	551	6.41	5.51

File: Data.125
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 9474
X Parameter: FL3-H (Log)

Log Data Units: Li
Patient ID: 139In
Case Number:
Panel:
Gate: G1
Total Events: 100
Y Parameter: FL2

Region	Events	% Gated	% Total
R1	9474	100.00	94.74
R2	305	3.22	3.05
R3	4496	47.46	44.96
R4	98	1.03	0.98
R5	4266	45.03	42.66
R6	362	3.82	3.62

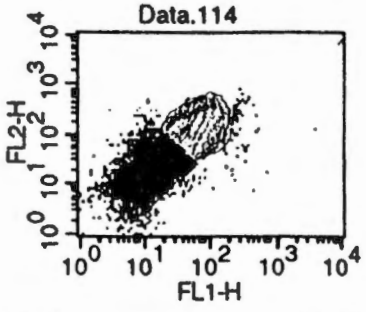
Figure 3-4C depicts CD28 (FL3) vs. B72 (FL2). R1= lymphocytes; R2=unstained;
R3=CD28+; R4=CD28+bright
R5=B72+bright; R6=B72+.



File: Data.111
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8262
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 135s5
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8262	100.00	82.62
R2	2028	24.55	20.28
R3	4151	50.24	41.51
R4	28	0.34	0.28
R5	23	0.28	0.23
R6	36	0.44	0.36
R7	2609	31.58	26.09

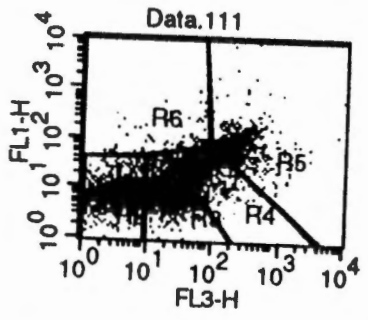


File: Data.114
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8433
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 138s5
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8433	100.00	84.33
R2	1382	16.39	13.82
R3	4020	47.67	40.20
R4	30	0.36	0.30
R5	8	0.09	0.08
R6	24	0.28	0.24
R7	3646	43.23	36.46

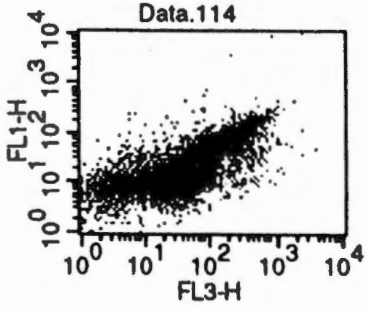
Figure 3-5: Representative sample of wild-type (134) and transgenic (139) spleen cells stained for CD28/B71/B72.
Figure A depicts B71 (FL1) vs. B72 (FL2). R1=lymphocytes; R2=unstained; R3=B72+; R4=B72+ bright; R5=B71+; R6=B71+/B72+; R7=B71+dull/B72+ (this population has been drawn in from the original printed blots because this population was in yellow, which did not photocopy).



File: Data.111
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8262
X Parameter: FL3-H (Log)

Log Data Units: Li
Patient ID: 135s5
Case Number:
Panel:
Gate: G1
Total Events: 100(
Y Parameter: FL1-

Region	Events	% Gated	% Total
R1	8262	100.00	82.62
R2	1192	14.43	11.92
R3	2752	33.31	27.52
R4	3048	36.89	30.48
R5	1135	13.74	11.35
R6	120	1.45	1.20

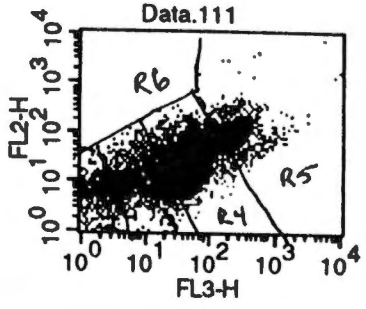


File: Data.114
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8433
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 138s5
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total
R1	8433	100.00	84.33
R2	920	10.91	9.20
R3	2275	26.98	22.75
R4	3552	42.12	35.52
R5	1644	19.49	16.44
R6	101	1.20	1.01

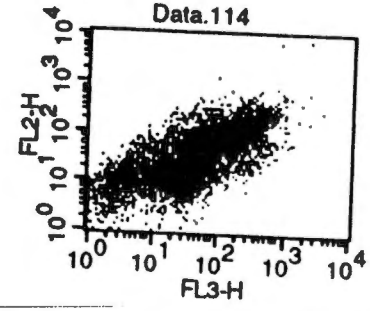
Figure 3-5B is CD28 (FL3) vs. B71 (FL1). R1=lymphocytes; R2=unstained; R3=CD28+dull; R4=CD28+; R5=B71+/CD28+; R6=B71+.



File: Data.111
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8262
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 135s5
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8262	100.00	82.62
R2	537	6.50	5.37
R3	1287	15.58	12.87
R4	5048	61.10	50.48
R5	931	11.27	9.31
R6	102	1.23	1.02

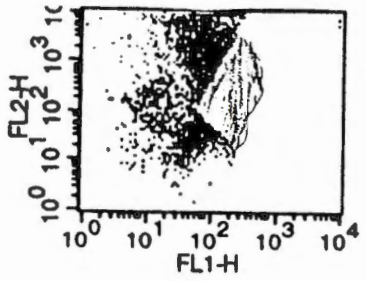


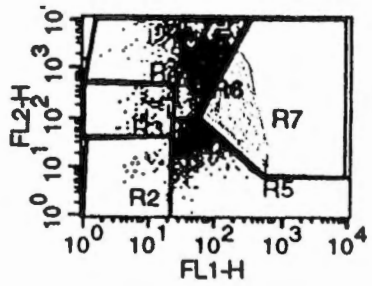
File: Data.114
Sample ID: 28/b71/b72
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8433
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 138s5
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8433	100.00	84.33
R2	358	4.25	3.58
R3	1000	11.86	10.00
R4	5116	60.67	51.16
R5	1550	18.38	15.50
R6	88	1.04	0.88

Figure 3-5C is CD28 (FL3) vs. B72 (FL2). R1=lymphocytes; R2=unstained; R3=CD28+dull; R4=CD28+; R5=CD28+bright; R6=B72+.





File: sw12296.103
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 2-Dec-96
Gated Events: 8073
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 90 s3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

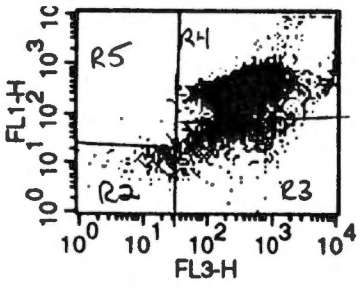
Region	Events	% Gated	% Total
R1	8073	100.00	80.73
R2	60	0.74	0.60
R3	197	2.44	1.97
R4	384	4.76	3.84
R5	610	7.56	6.10
R6	821	10.17	8.21
R7	6093	75.47	60.93

File: sw12296.101
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 2-Dec-96
Gated Events: 8151
X Parameter: FL1-H (Log)

Log Data Units: L
Patient ID: 88 s3
Case Number:
Panel:
Gate: G1
Total Events: 10(
Y Parameter: FL2

Region	Events	% Gated	% Total
R1	8151	100.00	81.51
R2	41	0.50	0.41
R3	91	1.12	0.91
R4	534	6.55	5.34
R5	945	11.59	9.45
R6	994	12.19	9.94
R7	5716	70.13	57.16

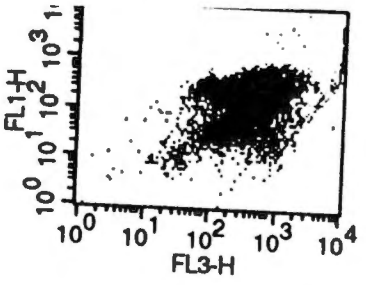
Figure 3-6: Representative sample of wild-type (90) and transgenic (88) spleens stained for mel-14/CD44/CD45RB.
Figure A depicts CD45RB (FL1) vs. PECD44 (FL2).
R1=lymphocytes; R2=unstained; R3=CD44+dull; R4=CD44+bright; R5=CD45RB+; R6=CD44+/CD45RB+dull; R7=CD44+/CD45RB+. (The R7 population has been sketched in from the original prints because R7 was in yellow and this did not photocopy.)



File: sw12296.103
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 2-Dec-96
Gated Events: 8073
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 90 s3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total
R1	8073	100.00	80.73
R2	290	3.59	2.90
R3	1313	16.26	13.13
R4	6319	78.27	63.19
R5	14	0.17	0.14

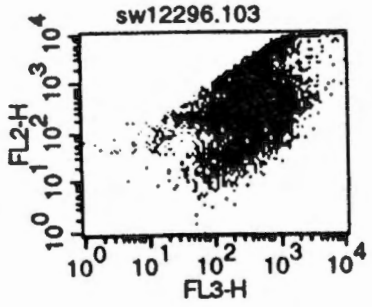


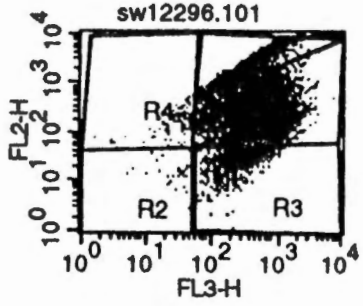
File: sw12296.101
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 2-Dec-96
Gated Events: 8151
X Parameter: FL3-H (Log)

Log Data Units: L
Patient ID: 88 s3
Case Number:
Panel:
Gate: G1
Total Events: 10(
Y Parameter: FL1

Region	Events	% Gated	% Total
R1	8151	100.00	81.51
R2	136	1.67	1.36
R3	2012	24.68	20.12
R4	5921	72.64	59.21
R5	25	0.31	0.25

Figure 3-6B is mel-14 (FL3) vs. CD45RB (FL1). R1=lymphocytes; R2=unstained; R3=mel-14+; R4=CD45RB+/mel-14+; R5=CD45RB+.





File: sw12296.103
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 2-Dec-96
Gated Events: 8025
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 90 s3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

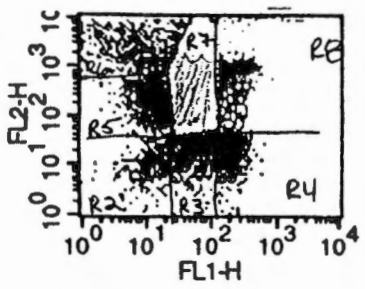
Region	Events	% Gated	% Total
R1	8025	100.00	80.25
R2	46	0.57	0.46
R3	744	9.27	7.44
R4	221	2.75	2.21
R5	5635	70.22	56.35
R6	1157	14.42	11.57

File: sw12296.101
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 2-Dec-96
Gated Events: 8130
X Parameter: FL3-H (Log)

Log Data Units: L
Patient ID: 88 s3
Case Number:
Panel:
Gate: G1
Total Events: 100
Y Parameter: FL2

Region	Events	% Gated	% Total
R1	8130	100.00	81.30
R2	36	0.44	0.36
R3	1084	13.33	10.84
R4	124	1.53	1.24
R5	5756	70.80	57.56
R6	1076	13.23	10.76

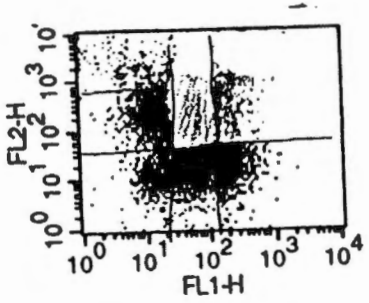
Figure 3-6C is mel-14 (FL3) vs. PECD44 (FL2). R1=lymphocytes; R2=unstained; R3=mel-14+; R4=CD44+; R5=CD44+/mel-14+; R6=CD44+bright/mel-14+.



File: Data.067
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8801
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 33ln3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8801	100.00	88.01
R2	402	4.57	4.02
R3	983	11.17	9.83
R4	757	8.60	7.57
R5	731	8.31	7.31
R6	638	7.25	6.38
R7	3535	40.17	35.35
R8	1764	20.04	17.64

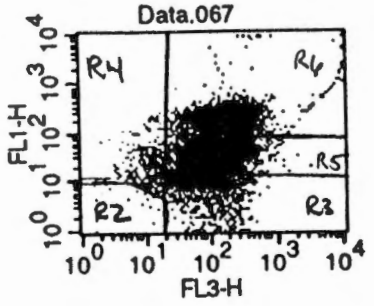


File: Data.070
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8856
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 136ln3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8856	100.00	88.56
R2	581	6.56	5.81
R3	827	9.34	8.27
R4	271	3.06	2.71
R5	1570	17.73	15.70
R6	852	9.62	8.52
R7	3416	38.57	34.16
R8	1364	15.40	13.64

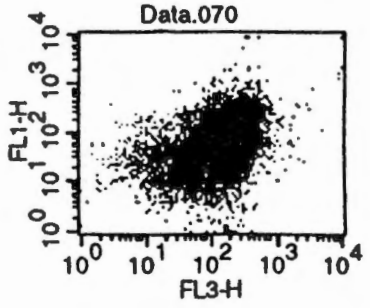
Figure 3-7: Representative sample of wild-type (33) and transgenic (136) lymph nodes stained for mel-14/CD44/CD45RB.
Figure A depicts CD45RB (FL1) vs. PECD44 (FL2). R1=lymphocytes; R2=unstained; R3=CD45RB+; R5=CD45RB+bright; R6= CD44+; R7=CD45RB+/CD44+; R8=CD45RB+bright/CD44+. (R7 has been sketched in because the it was yellow in the original prints and this did not photocopy.)



File: Data.067
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8801
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 33ln3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total
R1	8801	100.00	88.01
R2	358	4.07	3.58
R3	1960	22.27	19.60
R4	269	3.06	2.69
R5	4820	54.77	48.20
R6	1837	20.87	18.37

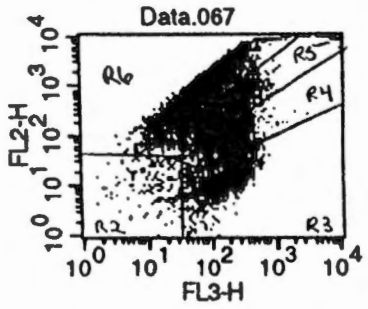


File: Data.070
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8856
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 136ln3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total
R1	8856	100.00	88.56
R2	329	3.71	3.29
R3	1499	16.93	14.99
R4	378	4.27	3.78
R5	5560	62.78	55.60
R6	1526	17.23	15.26

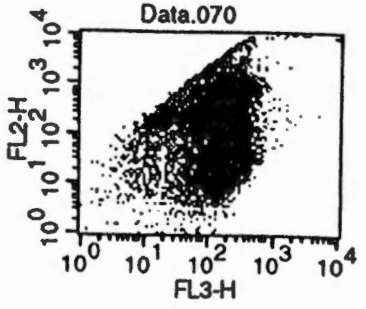
Figure 3-7B is mel-14 (FL3) vs. CD45RB (FL1). R1=lymphocytes; R2=unstained; R3=mel-14+; R4=CD45RB+; R5=mel-14+/CD45RB+dull; R6=mel=14+/CD45RB+bright.



File: Data.067
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8801
X Parameter: FL3-H (Log)

Log Data Units: Linear Values
Patient ID: 33ln3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8801	100.00	88.01
R2	205	2.33	2.05
R3	1545	17.55	15.45
R4	2333	26.51	23.33
R5	2817	32.01	28.17
R6	2004	22.77	20.04



File: Data.070
Sample ID: 14/44/45
Patient Name:
Tube:
Acquisition Date: 11-Jul-97
Gated Events: 8856
X Parameter: FL3-H (Log)

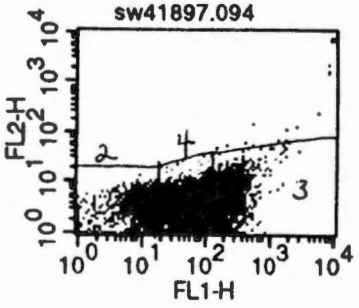
Region Statistics

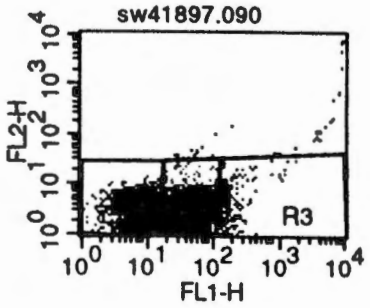
Log Data Units: Linear Values
Patient ID: 136ln3
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8856	100.00	88.56
R2	364	4.11	3.64
R3	2635	29.75	26.35
R4	2921	32.98	29.21
R5	2208	24.93	22.08
R6	935	10.56	9.35

Figure 3-7C is mel-14 (FL3) vs. PECD44 (FL2). R1=lymphocytes; R2=unstained; R3=mel-14+; R4=mel-14+/ CD44+dull; R5=mel-14+/CD44+bright; R6=CD44+.

Figure 3-8: Representative sample of thymocytes from wild-type (69) and transgenic (50) stained for CD43. Transgenic animals have a decrease in CD43+ cells. R1=lymphocytes; R2=unstained; R3=CD43+bright; R4=CD43+dull.





File: sw41897.094
Sample ID: cd43
Patient Name:
Tube:
Acquisition Date: 18-Apr-97
Gated Events: 8443
X Parameter: FL1-H (Log)

Log Data Units: l
Patient ID: 52t1
Case Number:
Panel:
Gate: G1
Total Events: 10
Y Parameter: FL

Region	Events	% Gated	% Total
R1	8443	100.00	84.43
R2	1203	14.25	12.03
R3	2609	30.90	26.09
R4	4550	53.89	45.50

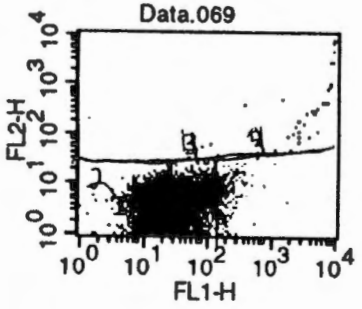
Region Statistics

File: sw41897.090
Sample ID: cd43
Patient Name:
Tube:
Acquisition Date: 18-Apr-97
Gated Events: 6465
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 26t1
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	6465	100.00	64.65
R2	1735	26.84	17.35
R3	258	3.99	2.58
R4	4363	67.49	43.63

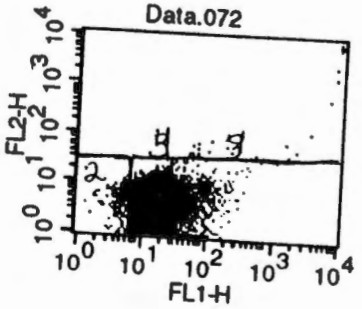
Figure 3-9: Thymocytes stained for CD43 showing wild-type (52) and transgenic (26) samples. Transgenic animals have a decrease in CD43+ cells. R1=lymphocytes; R2=unstained; R3=CD43+bright; R4=CD43+dull.



File: Data.069
Sample ID: cd43
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 8278
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 32t6
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	8278	100.00	82.78
R2	4550	54.96	45.50
R3	3261	39.39	32.61
R4	297	3.59	2.97



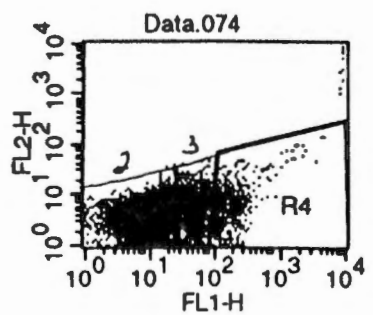
File: Data.072
Sample ID: cd43
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 4322
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 50t6
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	4322	100.00	43.22
R2	3238	74.92	32.38
R3	934	21.61	9.34
R4	74	1.71	0.74

samples had an increased number of CD43+ dull cells (Figure 3-10, Appendix 3-9) and lymph node samples had an upregulation (increase number of ligands per cell) of CD43+ cells compared to non-transgenic littermate controls (Figure 3-11, Appendix 3-10).

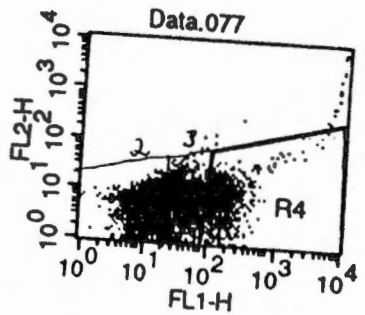
Figure 3-10: Splenocytes from wild-type (32) and transgenic (49) animals stained for CD43. Transgenic animals have an increase in CD43+ cells (Regions 3 and 4); R2=unstained.



File: Data.074
Sample ID: cd43
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 6815
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 32s6
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

Region	Events	% Gated	% Total
R1	6815	100.00	68.15
R2	4671	68.54	46.71
R3	2077	30.48	20.77
R4	707	10.37	7.07

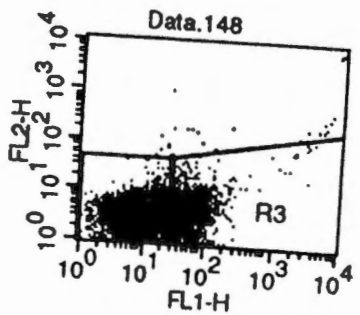


File: Data.077
Sample ID: cd43
Patient Name:
Tube:
Acquisition Date: 24-Jan-97
Gated Events: 7244
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 49s6
Case Number:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL2-H (Log)

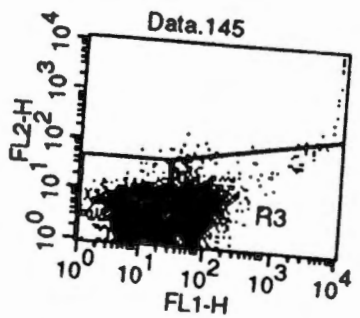
Region	Events	% Gated	% Total
R1	7244	100.00	72.44
R2	4483	61.89	44.83
R3	2658	36.69	26.58
R4	1079	14.90	10.79

Figure 3-11: Lymphocytes from wild-type (134) and transgenic (32) animals stained for CD43. Transgenic animals have an increase in CD43+ cells (R3); R2=unstained.



File: Data.148 Log Data Units: Li
Sample ID: cd43 Patient ID: 134In6
Patient Name: Case Number:
Tube: Panel:
Acquisition Date: 11-Jul-97 Gate: G1
Gated Events: 9265 Total Events: 100
X Parameter: FL1-H (Log) Y Parameter: FL2-

Region	Events	% Gated	% Total	X Mean	X Geo
R1	9265	100.00	92.65	38.77	
R2	7023	75.80	70.23	10.42	
R3	2259	24.38	22.59	81.05	



File: Data.145 Log Data Units: Li
Sample ID: cd43 Patient ID: 32In6
Patient Name: Case Number:
Tube: Panel:
Acquisition Date: 11-Jul-97 Gate: G1
Gated Events: 9243 Total Events: 100
X Parameter: FL1-H (Log) Y Parameter: FL2-

Region	Events	% Gated	% Total	X Mean	X Geo
R1	9243	100.00	92.43	64.56	
R2	5437	58.82	54.37	11.42	
R3	3798	41.09	37.98	90.34	

Chapter 5: Discussion

Based on the necropsy and histopathology results, combined with the *in vitro* assays and flow cytometry data, it appears that Wiskott-Aldrich syndrome protein (WASP) transgenic animals have an "activated" immune system. This poses two questions. One, is this an antigen dependent response (i.e. autoantigens, antigens in the environment) or an antigen independent response (i.e. overexpression of the transgene leads to a constitutively activated immune system? Two, if this is an antigen dependent response, is there an increase in "sensitivity/hyperresponsiveness" to antigens which causes the mice to have "activated" immune systems? If the transgenic mice were hyperresponsive, they could have amplified responses to antigens either due to a change in threshold/set point between tolerance, activation and hyperresponsiveness, or there could be a defect in antigen activation/apoptosis pathway such that the cells are activated but they are not regulated. As a result, the cells are not "turned off' once activated. In either event, (increased sensitivity to antigen or decreases in apoptosis), the immune system could potentially be hyperresponsive. These are the two questions which will be addressed. (1) Is the response antigen dependent or antigen independent? (2) Are the cells hyperresponsive? Based on the results of the data presented here, the wasp transgene appears to cause an antigen specific response. Secondly, the cells are activated but it is not clear at this time of there is a change in threshold/set point, such that the

amount of antigen required for a response is reduced. The data predominantly supports that the response is antigen specific. Functional assays demonstrate that background levels of proliferation and cytokine production are near zero and comparable to nonstimulated transgenic animals. This suggests that in a resting state, overexpression of the transgene does not result in upregulation of the immune responses. Therefore, WASP appears to cause upregulation/activation via an antigen specific response. Secondly, the transgene may upregulate the immune system by either causing a change in threshold such that cells are hyperresponsive to antigens or by decreasing the amount of apoptosis, or by potentiating responses once they are initiated through upregulation of the signaling pathway.

From the necropsy and histopathology data, WASP transgenic mice develop splenomegaly and lymphadenopathy which is characterized by marked germinal center hyperplasia. The question is what causes the germinal center hyperplasia? Why are the cells activated? What causes the cells to be activated? Is there a defect in T cell/B cell interactions or cell populations which lead to the hyperplasia? Are there defects/changes in the activation pathways which could explain these histological changes and overall activation of the immune system? Flow cytometry was used to determine if there were changes in cell populations and if there were changes in activation markers. From the flow cytoemtry daa, there is an increase in percentage of both T cells (CD3) and B cells (B220) present in both organs. B cells have an activated phenotype in that there is an

increased percentage of CD40+ cells. Its corresponding ligand, gp39/CD40L, present on T cells, appears to be expressed at levels comparable to the wild-type animals. If transgenic animals have an activated immune system and B cells are activated, one would theorize that the T cells would have an activated phenotype as well. Consequently, one would expect these T cells to have increased levels of CD40L compared to their nonactivated nontransgenic littermate controls because normally this ligand is expressed in very low levels on naive cells (Jaiswal, 1997; Roy, 1993). Only after the cells have been activated is it expressed in higher levels. This finding raises the question of whether the CD40/CD40L pathway is activated in the transgenics and/or whether this pathway is functional in the WASP transgenic animals. In order to determine if the pathway is functional, T cells from the transgenics may need to be activated with mitogens or antigens in order to determine whether they have the potential to express higher levels of CD40L on their cell surfaces (Roy, 1993). If, under *in vitro* conditions, the WASP transgenic T cells cannot be stimulated to increase expression of CD40L compared to non-transgenic littermate cells, this suggests a defect in the CD40/CD40L pathway. If the transgenic cells express CD40L at greater levels than the non-transgenic littermates, it suggests overexpression of WASP affects the CD40/CD40L pathway, but either this pathway is not being stimulated *in vivo* or there is some dysfunctional regulation that is preventing the upregulation of CD40L *in vivo*. If expression of CD40L is equal in transgenic and non-transgenic cells, one can say that the CD40/CD40L pathway is

functional but either this pathway is not being stimulated *in vivo* in transgenic animals or there is some other defect which is involved in activating the pathway *in vivo*. The latter is more likely because the transgenic B cells are activated as evidenced by increased numbers of CD40+, B71+ and B72+ cells. So, it suggests this pathway is stimulated, but there may be a defect in activating CD40L.

One pathway that does appear to be activated in these animals is the CD28/B7-1/B7-2 pathway. From spleen samples, the animals have a mild increase in numbers of CD28+ cells, as well as increased percentage of B cells which are B7-1+ or B7-2+. Cells from lymph nodes not only show an upregulation of CD28+, but there is also an increase in B7-1+, B7-2+, B7-1+/B7-2+ cells. Perhaps the overexpression of WASP protein has a more direct effect on the CD28/B7-1/B7-2 pathway versus the CD40/CD40L pathway. It could be that in these transgenics, the CD28/B7 signaling pathways function normally and whatever mechanism is activating the cells activates just this pathway, or that when the CD40/CD40L pathway is activated the B cells are upregulated, but there is a defect in CD40L upregulation, either in T cell/B cell interactions or T cell signaling events. From this data, it appears that the cells are activated. The CD28/B7 pathway is upregulated, and CD40 is upregualted in B cells, but there may be a defect in activating CD40L/gp39 in the T cells.

Analyzing the mel-14/CD44/CD45 cell surface activation markers, the data from this cobination of stains also suggests that a hyperresponsiveness is present. Spleen and

lymph node samples from WASP transgenic mice have increased percentages of cells that are CD45RB+. Spleen samples have increased number of CD45RB+ dull cells and samples from lymph nodes have an increase in CD45RB+ bright cells. The increase in CD45RB+ cells suggests an increase in effector cells and that the immune system is actively responding. There are several possible hypotheses which could explain the increases in CD45RB+ cells. (1) The transgenic animals continue to strictly see new antigen; therefore they continue to produce many CD45RB+ effector cells. (2) The transgenic animals cannot make memory cells, so they may be seeing the same antigen, but because they do not have a sufficient memory cell population, they are responding as though it is a primary immune response. (3) The transgenic animals have a change in threshold/set point such that less antigen is needed to mount a response. (4) There isn't a change in threshold, but due to the overexpression of WASP, the signals are potentiated and one sees an amplified immune response. (5) Overexpression of the transgene leads to constitutive upregulation of the immune response in an antigen independent manner. These possibilities will be addressed in order.

If the transgenics were seeing new antigen, the nontransgenic littermate controls would see the antigen as well and mount a similar response. Because the transgenics have a more "activated" immune system than wildtype littermates, this explanation is not likely. 1) The animals are not capable of accumulating a sufficient number of memory cells and therefore they are repeatedly undergoing the primary immune responses with

CD45RB+ effector cells. Perhaps that is why they have so many germinal centers and these contain many apoptotic cells -- transgenic animals cannot develop the memory cell population so they mount primary antigen responses to most antigens. If there is a problem at the level of producing memory cells, perphaps there is a defect in T cell/B cell interactions even at the level of the CD40/CD40L pathway, which prohibits the generation of sufficient numbers of memory cells. As a result, when the body comes in contact with this antigen again, there are inappropriate numbers of memory cells and the host develops predominantly a primary response again. This is a possible explanation.

2) If there is a change in the set point, this proposes that even though both transgenics and nontransgenic littermates are exposed to the same antigen, the transgenic animals require a lower dose of antigen to mount a response. This would result in marked germinal center hyperplasia and because this is an antigen dependent event, one would not see the spontaneous proliferation of cells or excess cytokine production by nonstimulated cells. Therefore, this hypothesis is a possible explanation

An alternative hypothesis for the increased number of germinal centers and CD45RB+ effector cells is that the mice are hyperresponsive. If there is an antigen dependent hyperresponsiveness, one could imagine that the cell needs multiple signals for activation, with WASP being one of those signals. The cell must still receive the initial signals for activation, but once WASP is activated, its overexpression leads to a heightened downstream response which results in hyperresponsiveness.

Alternatively, it is possible that the phenotype is the result of an antigen independent event. WASP is still involved in the cell signaling but its role is earlier in the transduction pathways or its role predominates such that overexpression of the transgene results in a direct upregulation of downstream events which leads to a constitutive and marked upregulation of the immune response. As a result, there is marked germinal center hyperplasia directly due to overexpression of the transgene and not due to an antigen dependent event. If this were true, then one would expect to see spontaneous proliferation of the cells on blastogenesis assays as well as high levels of cytokine production in nonstimulated samples. Because one does not see either of these, it suggests that WASPs effects are not due to an antigen independent phenomena.

In order to address these possibilities, the data thus far needs to be considered and additional experiments need to be proposed and conducted. Based on the flow cytometry data, it has been demonstrated that there are increased numbers of B and T cells in the WASP transgenic spleens and lymph nodes and that these cells reveal activation of both the CD28/B7 pathway and CD40 of the CD40/40L pathways. *In vivo* challenges of naive and immunized animals could help to determine that the activation is inducible versus constitutive. If this response is antigen independent the naive animals will have upregulation of cell surface markers before being immunized. Following stimulation, there may be some additional upregulation of the markers. Comparatively, if the process is antigen dependent, lymphocytes from naïve mice will have normal to slightly

increased expression of markers before immunization, and cell surface marker expression will markedly increase after animals are challenged.

During these in vivo challenges, serology and other functional assays can be conducted to address these proposed explanations. Serology can be used to assess *in vivo* B cell responses. Based on analysis of IgA, IgM and IgG from lines Tg5550 and Tg5562, it appears that transgenic animals have elevated levels of IgG, but comparable levels of IgA and IgM. For animals which have such a marked increase in germinal centers, containing many apoptotic cells, compared to non-transgenic littermate controls, one would expect that they would have marked increases in serum levels of immunoglobulins, especially considering that the transgenic animals have increased numbers of B cells and T cells with activated phenotypes (i.e. CD28/B7/CD40). The elevation in IgG vs. IgA and IgM suggests that there is at least some degree of class switch occurring, but it may be insufficient. Perhaps there is a defect in the ability to produce functional germinal centers. Under these given conditions, germinal centers could be generated but there is a defect at either the level of antigen presentation, proliferation, somatic mutation, class switch or memory cell generation. As a result, the germinal centers are dysfunctional. Perhaps that is why there are marked number of apoptotic cells in the germinal centers. One explanation for this could be that overexpression of WASP in the T cells, alters T cell B cell interactions, which in turn affect B cell responses. For example, overexpression of WASP could alter T cell

signaling resulting in changes in CD40L expression which, because of an inadequate CD40/CD40L interaction between B and T cells, leads to inadequate B cell responses. As a result, despite the marked increases in germinal centers, there are not marked increases in immunoglobulin production. Testing expression of CD40/CD40L and immunoglobulin levels with *in vivo* challenge experiments could determine whether these results are consistent. This would support explanation (2). If the animals produce adequate responses or marked increase in immunoglobulin production, this supports explanations 3,4,or 5. If WASP constitutively upregulates the immune system, then the naïve animals should have elevated immunoglobulin levels. If necessary, one could immunize with different concentrations of antigen. If there were a change in the setpoint/threshold, one would expect to see increased responses with increasing levels of antigen. Whereas if (4) is accurate, once a minimum dose of antigen is given, there should be increased levels of immunoglobulin because of WASP's effects on potentiating responses.

Even from these *in vivo* experiments, it may be difficult to determine which explanation is correct. One can do in vitro blastogenesis assays designing a dose response curve. If the transgenic animals have a change in set point, one should see increased prolifearive indices compared to wild-type animals regardless of the dose. Although at high doses of antigen, the dose response curve may level off. By comparison, if the animals have an upregulation of the signal cascade from some point further downstream

in the signal cascade, the responses may be very similar to what one would find with a change in set point. If the antigen is the limiting factor, then once a minimum amount of antigen is present, one may see the same peak of proliferation responses regardless of the amount of antigen. However, it is possible that the dose contributes to the response and the dose response curve may be similar to what would find with changes in set point. Because it may be difficult to differentiate which mechanism is correct, one may need to use cell signaling assays to determine if WASP transgenic animals upregulate the signaling pathway constitutively, and if so, from what point in the pathway does WASP has its effect?

Finally, several *in vitro* assays have been done to assess T and B cell function. Again, these results can help to assess whether the phenotype is due to an antigen independent or dependent response. For point of reference, by flow cytometry, there were no changes in CD4:CD8 ratios or distributions from transgenic thymus samples. First, blastogenesis studies were conducted for both B and T cells. Con A and anti-CD3 were used to look at T cell proliferation. With Con A, the TCR does not have to be cross-linked where as anti-CD3 is a more physiological mitogen. Thymocytes from transgenic animals produced mildly increased stimulation indices to Con A compared to wild-type animals. Responses to anti-CD3 have not been done due to limited samples. Both spleen and lymph node samples produced increased proliferative responses to Con A and anti-CD3. (Table 3-3) This suggests that transgenic T cells from all lymphoid organs are capable,

when stimulated, of producing elevated proliferation responses. Non-stimulated samples did not appear to have higher backgrounds, or higher spontaneous proliferation, which suggests that despite constitutive overexpression of the transgene, there is not a marked increase in baseline levels of proliferation. This supports the idea that even though there may be increased upregulation of the downstream events of WASP signaling, there needs to still be an inciting antigen or artificial stimulation/activation of the cell. Activation of the immune system is an antigen dependent event. These results suggest that not only are the WASP transgenic T cells capable of producing adequate responses, but they are hyperresponsive. These results do not contradict the findings that we have a strong phenotype in animals housed in a conventional setting. These animals are exposed to antigens (i.e. parasites, environmental antigens) in their normal environment. These daily antigens, along with the hyperresponsiveness or a change in threshold of the immune cells, may be sufficient means to produce this phenotype. It is expected that when C-sectioned animals are examined, the phenotype will be present, but it will be less severe because there are fewer environmental antigens.

To assess B cell responses, LPS was used (10ug/ml) as a mitogen. Proliferative response to LPS is a T cell independent B cell event, thus any effects of the transgenic T cells should not affect the B cell proliferation responses. B cells from both spleen and lymph node samples produced increased stimulation indices compared to non-transgenic littermate controls. (Table 3-3) Baseline levels of proliferation were comparable,

suggesting tha this is an antigen dependent event. Again, when stimulated, WASP transgenic B cells are capable of proliferating, and they are in fact hyperresponsive. The question remains, why are the B cells hyperproliferative? These transgenic mice were generated using a T cell specific promoter. One explanation could be that the promoter is leaky, such that B cells may also express the transgene at some low level. As a result, when cells are stimulated they are hyperresponsive. RNase protection experiments will be conducted to determine whether B cells express the transgene, and if so, to what degree? Through RNase protection experiments, it is expected that we will be able to demonstrate that transgenic T cells have the highest level of transgene expression and transgenic B cells have a very low, if any, quantifiable levels of expression, non-transgenic T cells have endogenous expression only and non-transgenic B cells have endogenous expression. If there is expression of the transgene by the transgenic B cell population, RNase protection assays will allow us to quantitate these levels. If contaminated cells are present, it is expected that the levels of the transgene expression will be the same as or slightly higher than endogenous expression, where as if the promoter is leaky, expression of the transgene will be moderately greater than endogenous expression but less than that of the population of transgenic T cells. If there is still a problem of discerning whether there is contamination, the samples will also be probed with a TCR probe to determine whether there are any T cells in the B cell samples. Some preliminary work has been done by RT-PCR examining B and T cell

populations. At very high cycle conditions, there was a trace band in the B cell sample, but this was minimal compared to the band present in the T cell samples. It is thought that there may be some T cell contamination of the B cell population which was isolated by Dyna-beads (Witonsky, unpublished data). Alternatively, flow cytometry can be done on the separated samples to assess purity. If needed, the separated B cells can be treated with anti-thy1 and complement to lyse any T cells before RNase protection or RT-PCR is performed.

Based on blastogenesis results, it appears that both B and T cells are hyperresponsive to mitogens. Subsequently, cytokine production by T cells was analyzed to determine in there were any correlations between increased blastogenesis results and cytokine production. Samples from thymus, spleens and lymph nodes were harvested, plated and stimulated as described above. Supernatants were collected and tested for IL-2, IL-4 and IL-6 production. Both spleens and lymph nodes had elevated IL-2 production from 16-18hr stimulated samples, but not non-stimulated samples. This suggests that baseline levels of cytokines are comparable, but that when WASP transgenic cells are stimulated with Con A, they produce increased levels of IL-2. This correlates with blastogenesis results in that T cell proliferation is regulated by IL-2 production. If there is increased proliferation by T cells then one would expect elevated levels of IL-2.

From the information presented here, it is clear that both T cells and B cells independently are capable of producing adequate responses *in vitro*, and they are in fact

hyperresponsive, when stimulated. If the cells are not stimulated, their proliferation indices and cytokine production are comparable to the wild-type animals. This suggests that these responses are antigen/mitogen dependent. In order to see the effects of overexpression of *wasp*, the transgenic cells must first be stimulated in some manner. Even with high levels of constitutive *wasp* expression, non-stimulated cells do not appear to be hyperresponsive *in vitro*. In order to confirm that the hyperresponsiveness is antigen dependent, and to determine whether the animals can mount appropriate T cell dependent B cell responses, *in vivo* experiments will need to be done. The *in vivo* challenges of LPS (Foster, unpublished studies) and rabbit immunoglobulin (Ig), as described earlier, will determine T cell independent B cell responses and T cell dependent B cell responses, respectively. If overexpression of the transgene results in constitutive upregulation of the immune system, then one expects higher baseline immunoglobulin levels copared to the wildtype animals. Immunoglobulin levels in immunized animals will be at least as high as the baseline levels. If the effects of overexpression of the WASP protein are antigen dependent, one would expect increased immunoglobulin responses to T cell dependent events. Independent B cell responses, if elevated, are suggestive either of transgene expression in the B cells or some compensatory upregulation of the B cells by the host. If B cell function is normal, then this suggests that transgene expression is limited to T cells. These *in vivo* experiments will provide additional data needed to: 1) substantiate that activation of the immune

system is an antigen dependent event 2) determine which proposed mechanism of activation is accurate. It is possible that even with these experiments one will not be able to differentiate between a) change in setpoint/threshold b) upregulation from WASP and further downstream. Cell signaling experiments may be needed to elucidate WASPs function and mechanism of action.

Finally, because there is considerable controversy about the association of CD43 and WAS patients, in that some studies have reported defective expression of CD43 and/or alterations in CD43 stimulated proliferation responses, cell surface expression of CD43 was analyzed to ascertain if there were any correlations which could be drawn between overexpression of WASP protein and CD43. There are two different isoforms of CD43 present at different stages of T cell development and activation. One is the 115kd (S7) form present predominantly on resting cells. The 115kd isoform has a considerable number of O-linked carbohydrates, which give it a negative charge, and it may be involved in cell-cell repulsion. The other is the activated isoform, 130kd (B11). All thymocytes are strongly positive for the 115kd resting form. The 130kd form is found on double negative (DN), double positive (DP) and some single positive CD8+ (SP 8+) cells (10-15%+), but not on CD4+ cells. Peripherally, both resting CD4+ and CD8+ cells express high uniform levels of the 115kd isoform. With activation, CD8+ cells downregulate the 115kd isoform and upregulate the 130kd form. In contrast, CD4+ cells upregulate both the 115kd and 130kd isoforms (Ellies, 1996; Jones, 1994).

The different roles of these CD43 isoforms are not clear because of the differential expression of them during thymic selection and activation, and this role may be due, in part, to their adhesive/aggregation properties. Recently, it has been demonstrated that both CD45 and CD43 have N-acetyllactosamine sequences, which bind galectin-1 present on thymic epithelial cells (TECs). If CD43 expression is altered, this could effect the interaction of these T cells and TEC's or other cells expressing galectin. In addition, galectin-1 is capable of inducing apoptosis and altering the O-glycan elongation of CD43, which affects the ability of CD43 to interact with galectin-1, and this results in potentiating apoptosis (Ellies, 1996; Barum, LG, 1995; Perillo, 1995). In correlation, CD43 may be involved in thymic selection events, including apoptosis as well as a role in regulating adhesion properties in both resting and activated cells. Supportive of this role is that CD43 is associated with the cleavage furrow during cytokinesis, and it is linked with the ezrin-radixin-moesin (ERM) family members and actin filaments directly or indirectly (Yonemura, 1993). If CD43 expression is altered, or actin polymerization is affected, CD43 mediated immune functions are impaired. Animals which overexpress CD43 under an immunoglobulin promoter have increases in B lymphocyte aggregation and decreased DTH responses (Ostberg, 1996). CD43 knock-out animals have enhanced proliferation responses to Con A, CD3 and allostimulation. These animals also had increased cytotoxic T lymphocyte (CTL) responses to vaccinia virus, but yet they also had an increased viral load. Manjunath et al. concluded that CD43 negatively regulates T

cell activation and adhesion. In analyzing the results and conclusions from these experiments, it is apparent that CD43 has a role in proliferation and adhesion but the exact mechanism is not clear (Manjunath, 1995, Mc Farland, 1995).

Recently WASP has been associated with actin polymerization from both morphological and cell signaling experiments. In transfection studies, it has been demonstrated that overexpression of WASP alters actin polymerization and confers a more rounded cell morphology. In this same study, WASP was immunoprecipitated with cdc42Hs and it was also shown to interact with signaling molecules, rho and rac; WASP did bind rho with a higher affinity than rac (Symons, 1996). Considering these experiments along with the observations that some WAS patients have small platelets and lymphocytes with blunted microvilli and variable CD43 expression and proliferation responses, it was elected to examine CD43 in WASP transgenic mice. As an initial starting place, WASP transgenic lymphocytes were analyzed by FACS analysis for CD43 cell surface expression.

For these studies, only the resting 115kd antibody was available at the time. As analyzed by flow cytometry, it appears that these animals do have altered cell surface expression of CD43. This could be a direct or downstream effect of the transgene expression. From thymus samples, there is a decrease in the CD43+ dull population of cells. In spleen and lymph node samples, there is an increase in CD43+ cells, which

affects both the dull and bright populations. This suggests an actual increase in the number of CD43+ cells and not just upregulated expression on the same number of cells.

The decrease in dull CD43+ thymic cells may be suggestive of a decrease in negative charge by the cells and those cells may have a decrease in repulsive charges or be more likely to aggregate. Another possibility is that there is a decrease in the 115kd isoform of dull cells, but there could be an increased number or intensity of cells with the activated isoform (130kd) present. Follow up studies need to be done on thymus, spleen and lymph node samples to determine if there are changes in the activated isotype as well. In addition, because of he association between altered CD43 expression and changes in adhesion properties (Ostberg, 1996), aggregation studies should be conducted to determine if there is a correlation between decreased CD43+ dull cells and increased aggregation properties.

These future experiments will help to determine the role, if any, of WASP and CD43 in the thymus, because at this point the significance of these findings is not yet clear. Grossly, WASP transgenic animals develop thymic atrophy as they are aged, though histologically, the thymuses from younger animals appear normal. Based on flow cytometry studies, CD4+ and CD8+ populations and distributions appear normal, thus thymic selection in the young mice may not be significantly affected. In the older mice, the CD4:CD8 ratios and distributions are similar to the non-transgenic animals. This suggests that there is a decrease in all populations of cells, which doesn't alter the

CD4:CD8 ratios; all populations are affected equally. There are just fewer total numbers of cells present in the older transgenic animals compared to the non-transgenic littermate controls. In analyzing the blastogenesis results, the cells are hyperproliferative in response to Con A. So it appears as though the transgene does affect thymocyte function to some degree, although it may not affect selection, based on the CD4: CD8 staining. Subset analysis using V_B markers to assess thymic selection has not been done at this time. These additional studies will help to elucidate the role of CD43 in the thymus.

WASP transgenic spleens and lymph nodes have increased numbers of CD43+ cells and upregulation of CD43 on existing cells. Based on the studies by Ellies et al (1996), this would suggest a possible activated status of the immune system. Again, now that the antibody for the activated 130kd isoform is available, experiments need to be repeated to show whether the 130kd isoform is also upregulated. Aggregation, actin polymerization and electron micrograph (EM) studies also need to be conducted in the future to determine if overexpression of the WASP protein affects these functions, and if so, how do these results correlate with the proliferation and cytokine experiments. These experiments will help to determine how CD43 and WASP are associatedand how this interaction affects immune cell function, with respect to cell morphology, adhesion and aggregation properties. How do these protein molecules affect immune cell responses, and if this relationship is altered by overexpression of the *wasp* gene, what are the affects on immune function?

To this point we have determined that overexpression of the normal WASP protein in mice results in splenomegaly and lymphadenopathy due to increased germinal center activity. As such, there is an increase in T and B cells within these lymphoid organs. These cells appear to be activated in that they have increased numbers and upregulated expression of CD28 and CD40, B7-1 and B7-2. In addition, they have alterations in CD43 expression compared to the nontransgenic littermates. *In vitro* functional tests demonstrate that both B and T cells are hyperproliferative, and stimulated T cells produce increased amounts of IL-2. Based on these results and that background levels of proliferation and IL2 production are comparable to nontransgenic littermate controls, it appears as though the transgene causes an antigen dependent overactivation of the immune system. *In vivo* challenge experiments need to be conducted in order to determine whether the hyperresponsiveness is due to 1) inability to produce memory cells, due to a defect in T cell/ B cell responses. 2) change in threshold for antigenic response. 3) no change in set point, but upregulation of WASP downstream events which results in amplified immune responses.

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APPENDIX

	7/11/97	7/11/97	4/26/97	4/26/97	1/24/97	1/24/97
CD3/B220	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
unstained	16.49	12.72	6.19	8	12	8.4
CD3	13.16	12.59	14.77	14.63	21.47	32.09
3+ bright	19.74	21.73	26.73	27.1		
220+ bright	23.42	24.34	5.61	16.38	23.83	30.46
220+	2.98	4.26	1.75	2.76		

Appendix 3-1: Raw data for flow cytometry: FITC-CD3/PE-B220 spleens. The top row refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

	7/11/97	7/11/97	4/26/97	4/26/97	1/24/97	1/24/97	12/22/96	12/22/96
CD3/B220	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
unstained	1	0.67	6.88	5.3	1.55	1.01	2.54	1.47
CD3	44.54	31.76	22.53	26.34	58.27	60.69	38.62	32.26
220+ dull	5.04	6.11	1.49	3.65	4.6	5.56	5.38	4.54
220+ bright	8.18	13.6	4.6	7.93	9.9	15.7	12.31	19.49
3+/220+	25.24	32.51	28.34	25.62	4.45	4.49	13.02	16.32

Appendix 3-2: Data from flow experiments for FITC-CD3/PE-B220 lymph nodes. The top row refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

	1/24/97	1/24/97	1/17/97	1/17/97	4/18/97	4/18/97	7/11/97	7/11/97
CD40/CD40L (gp3	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
unstained	31.21	41.38	7.31	4.64	8.37	9.48	14.42	12.16
CD40+	13.4	22.52	16.04	15.24	39.5	30.15	32.59	31.38
CD40+int.	1.8	7.07	58.02	63.5	26.09	25.31	22.51	24.33
CD40+ bright	3.82	8.34	2.11	0.87	14.52	18.36	3.86	4.96
CD40L+	0.33	0.37	0.14	0.09	0.32	0.4	0.18	0.15

Appendix 3-3: Data from flow cytometry experiments from lymph node samples stained for FITC-CD40/ PE-CD40L. The top row refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

CD28/B71/B72	4/18/97	4/18/97	7/11/97	7/11/97	12/22/96	12/22/96	12/2/96	12/2/96	1/17/97	1/17/97
	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
B71/B72										
B72+	0.12	0.57	16.76	14.15	5.61	7.2	4.18	5.21	2.1	0.71
B71+	7.4	10.52	20.37	26.8	0.02	0.03	0.2	0.3		
B71+dull/B72+	8.92	8.87					2.66	2.23	47.75	26.65
B71+brght/B72+	76.71	21.65	17.22	20.81	11.27	13.21	74.64	72.28	27.91	54.35
B71+int./B72+	7.06	7.35								
CD28/B71+										
CD28+int.			41.48	38.73						
B71+	3.63	5.85			18.73	23.39	0.14	0.15	0.04	0.05
B71+bright					2.26	2.41				
B71+/CD28+dull					3.44	4.28	0.68	5.11		
CD28+	7.14	6.8	21.65	20.66	0.49	0.51	1.21	15.11	32.38	9.87
B71+/CD28+	0.44	0.47	4.18	6.38	0.77	0.76			18.16	55.25
B71+/CD28bright	40.76	43.45								
CD28+ bright			16.35	19.92			76.18	70.2	38.43	22.46
B72+/CD28+										
CD28+dull	7.48	9.89	48.21	44.71	32.21	27.33				
CD28+					26.74	32.76	15.39	22.86	16.44	6.06
CD28+bright	10.66	13.11	1.01	1.69	2.73	2.61				
CD28+/B72+			56.83	35.98	3.26	4.33				
B72+	0.7	1.62	4.98	4.66	7.03	8.19	0.2	0.1	0.54	0.26
CD28/B72+dull	32.41	31.03					68.38	61.67	73.63	78.26
CD28+/B72+brigh	0.53	0.87					3.35	3.19		

Appendix 3-4: Data for flow cytometry from lymph nodes stained for biotinylated-CD28/FITC-B71/PE-B72. The top row refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

	7/11/97	7/11/97	1/24/97	1/24/97	12/2/96	12/2/96
CD28/B71/B72	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
B71/B72						
B72+dull	35.44	34.34				
B72+bright	0.41	0.36	1.06	1		
B71+	0.2	0.17	11.67	11.71	0.2	0.3
B71+/B72+	0.51	1.12	0.75	0.5	4.18	5.21
B71+dull/B72+	26.22	29.41				
B71+/B72+dull					2.66	2.33
B71+/B72+dull					74.64	72.8
CD28/B71						
CD28+dull	22.96	20.2	44.44	54.98	12.1	15.11
CD28+	27.89	28.55	13.62	16.89	76.18	70.2
CD28/B71+	14.5	16.16	4.53	5.55	0.675	5.11
B71+	0.93	1.22	0.05	0.13	0.135	
CD28/B72+						22.86
CD28+dull	12.47	12.21	52.46	64.35	15.39	
Cd28+int.	42.65	41.16		6.2		
CD28+/B72+	14.15	15.54	4.21	5.12		
B72+	0.77	0.76	0.02	0.04	0.2	0.1
CD28+bright			4.81	6.2		
CD28+/B72+dull					68.38	61.67
CD28+/B72+bright					3.35	3.19

Appendix 3-5: Data for flow cytometry from spleens stained for biotinylated CD28/FITC-B71/PE-B72. The top column refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

4/26/97	12/2/96	12/2/96	10/30/96	10/30/96	1/17/97	1/17/97
T/+	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
2.07	0.28	0.21				
	8.8	8.24	5.18	4.77	3.56	1.86
12.13	3.18	4.15				
6.71			10.06	11.31	30.26	30.51
7.06	17.48	18.79	25.28	23.83	7.04	7.6
21.05	54.8	52.01	29.59	31.95	8.16	6.68
					34.41	30.61
13.08			4.48	4.12	15.11	13.8
	0.35	0.35			10.5	8.61
			16.69	14.85	2.11	1.32
2.44	0.65	0.53	14.47	19.08		
39.28	16.56	20.52	11.6	12.06	38.13	34.03
	66.58	62.33	23.44	21.98	19.88	21.44
13.94	22.51	25.78	11.38	10.5	18.57	19.05
5.87	2.12	2.08	31.49	43.92	3.1	1.98
20.91	43.92	40.86	13.17	11.67	4.67	40.1
12.72	15.54	14.2	26.5	28.08	19.06	14.33

Appendix 3-5 continued.

mel-14/CD44/CD45R	12/2/96	12/2/96	7/11/97	7/11/97	4/18/97	4/18/97
	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
CD44/CD45RB						
CD44+dull	1.68	1.96	3.78	6.51	4.74	5.81
CD44+bright	5.74	6.38	3.54	3.25	2.82	3.03
CD45RB+	6.3	7.45	12.94	15.29	9.04	12.18
CD44/CD45RB+dull	8.82	9.78	15.07	16.89	4.02	3.32
CD44/CD45RB+	60.5	56.35	33.28	27.33	48.61	40.33
CD45RB+bright			4.25	2.85	3.11	1.83
mel-14/CD45RB+						
mel-14+	15.34	18.62	25.03	33.28	4.09	4.53
mel-14+/CD45RB+	63.97	58.85	15.75	13.71	54.66	50.17
CD45RB+	0.12	0.18	26.03	19.06	44.41	34.69
					14.23	18.19
CD44/mel-14					35.96	27.25
mel14+	8.04	8.78	10.6	11.98	9.56	10.94
CD44+	1.84	1.99	26.86	23.53	25.9	27.39
CD44+dull/mel-14+	56.51	55.07				
CD44+bright/mel-14	14.18	13.01				
CD44/mel-14+dull			21.76	20.87	32.96	26.17
CD44+/mel-14+bright			10.6	13.4	6.05	5.35

Appendix 3-6: Data from spleens stained for biotinylated mel-14/PE-CD44/FITC-CD45RB. The top column refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

mel-14/CD44/CD45RB	7/11/97	7/11/97	4/18/97	4/18/97	12/12/96	12/12/96	1/24/97	1/24/97	4/26/97
	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)
CD44/CD45RB									
CD44+dull	8.38	9.32	3.91	4.52	5.46	7.1			1.42
CD44+bright	5.26	4.41	5.63	6.54	1.52	1.29	7.39	5.66	
CD45RB+dull	9.68	11.93	13.42	11.19	24.67	25.81			8.57
CD45RB+bright	7.38	8.95	6.5	5.46	10.6	12.68	7.73	9.53	6.58
CD44/CD45RB+dull	36.02	30.57	29.82	22.86			5.39	5.08	5.96
CD44/CD45RB+bright	16.22	19.64	22.22	27.91	13.47	14.86	39.08	39.54	20.08
mel-14/CD45RB									
mel-14+	19.91	17.63	4.53	5.02	16.64	19.34	7.94	6.79	10.79
mel-14+bright					8.59	9.58			
CD45RB+dull	4.12	2.88	10.4	10.29					
CD45RB+bright					12.77	14.3	5.43	5.16	2.59
mel-14+/CD45RB+dull	49.86	49.34	67.02	64.65	18.48	19.2	45.85	47.29	34.97
mel-14+/CD45RB+brig	17.16	21.14							
mel-14/CD44									
mel-14+	16.7	22.68	15.15	12.95	4	4.69	8.5	10.14	11.06
CD44+	16.15	13.13	27.15	32.85	10.55	12.5	15.53	12.04	4.5
mel-14/CD44+dull							36.39	38.31	17.3
mel-14/CD44+bright			38.95	32.75					12.62
CD44+/mel-14+dull	25.62	26.84			32.3	34.44			
CD44+/mel-14+bright	25.44	23.99			11.76	15.37			

Appendix 3-7: Data from lymph nodes stained for biotinylated mel-14/PE-CD44/FITC-CD45RB. The top column refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

Appendix 3-8: Flow data from thymocytes stained for FITC-CD43. The top column refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

	7/11/97	7/11/97	4/18/97	4/18/97	1/24/97	1/24/97
	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
CD43						
unstained	43.87	42.99	10.05	13.84	51.26	55.51
CD43+dull	51.76	45	28.27	8.82	30.52	10.3
CD43+ bright			47.64	51.06	3.2	0.64

Appendix 3-9: Flow data from spleens stained for FITC-CD43. The top column refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

	1/24/97	1/24/97	10/30/96	10/30/96	7/11/97	7/11/97	4/26/97	4/26/97
CD43	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
unstained	45.9	41.74	47.68	47.05	53.25	61.71	14.77	15.56
CD43+ dull	22.41	25.07	16.37	23.22	27.1	30.26	20.1	23.62
CD43+ bright	6.88	9.01					16.69	15.35

Appendix 3-10: Flow data from lymph nodes stained for FITC-CD43. The top column refers to the date when the samples were collected. Transgenic samples are marked by (T/+). Wild-type samples are marked by (+/+). The left lane denotes the different possible populations based on the stains used.

	1/24/97	1/24/97	4/18/97	4/18/97	7/11/97	7/11/07
CD43	(+/+)	T/+	(+/+)	T/+	(+/+)	T/+
unstained	49.72	49.48	9.25	6.36	66.24	59.39
CD43+ dull	28.33	30.63	57.2	48.16	25.66	31.49
CD43+ bright			27.12	34.93		

PART IV: OVEREXPRESSION OF WASP PROTEIN ASSOCIATED WITH DIFFERENTIAL PHOSPHORYLATION OF MULTIPLE PROTEINS

Chapter 1: Abstract

Wiskott-Aldrich syndrome is characterized by a triad of clinical signs including thrombocytopenia, eczema and a progressive B and T cell mediated immunodeficiency. The pathophysiology of the disease is not well understood, and the function of the gene has not yet been elucidated. Recently, the WASP protein has been associated with several molecules in the signal transduction cascade, and it has been correlated with a role in actin polymerization. Here we report that overexpression of the *wasp* gene results in the altered phosphorylation of several proteins which may contribute to the phenotype seen in these transgenic mice. Identification of these proteins will not only help to explain the phenotype of the WASP transgenic mice but also to elucidate the role of this protein in the pathophysiology of Wiskott-Aldrich syndrome.

Chapter 2: Introduction

Wiskott-Aldrich syndrome is an X-linked recessive disorder for which the gene has recently been identified. The syndrome is characterized by a triad of clinical signs including thrombocytopenia, with small platelets, eczema, and a progressive immunodeficiency. The immunological defects vary considerably between patients based on the nature of the mutation as well as other unknown factors. In some patients, B and T cell function appears to be normal, whereas in other patients with the same genetic mutation, they are severely immunocompromised. Patients who survive are markedly susceptible to lymphomas, including those associated with EBV. They have >100X incidence for the development of tumors, and they are also at risk for the development of autoimmune diseases, including renal disease, autoimmune hemolytic anemias (AIHA), vasculitis and arthritis.

The pathophysiology of this syndrome is not understood. It is confounded by the variations in the severity and presentation of the disease, even among patients with the same genetic defect. Recently, it has been demonstrated that overexpression of WASP *in vitro* alters actin polymerization.WASP was also immunoprecipitated with the GTP associated signaling molecule cdc42, and two other yet unidentified proteins. Other

studies have associated WASP with other kinases, including nck, *fyn* and *Itk* (Cory, 1996; Symons, 1996). Here we demonstrate that overexpression of the *wasp* transgene alters phosphorylation of several proteins as detected by signal transduction assays. Even in the resting state, there is the differential phosphorylation of several proteins which could play a significant role in the pathophysiology of the phenotype exhibited by these mice. Equally important, is that these changes in cell signaling may help to localize WASP within the signal transduction cascade, and they may help to elucidate the role of this protein in Wiskott-Aldrich syndrome.

Chapter 3: Materials and Methods

Maintenance of animals and transgenic lines: Animals are maintained on an inbred FVB stock by mating heterozygous transgenic animals x non-transgenic FVB's. All mice were housed in a conventional environment, and fed a standard laboratory diet and water *ad libitum*. Animals are genotyped by PCR using a 5' primer specific for the transgene, within the multiple cloning site of the promoter, and a 3' primer in the 5' end of the mouse *wasp* cDNA.

Preparation of samples for assays: At necropsy, lymphoid organs were collected and put in 5ml aliquots of complete media (GIBCO RPMI supplemented with 5% heat inactivated fetal calf serum (FCS- Intergen), 2% GIBCO antibiotic/antimycotic (penicillin/streptomycin and amphotericin B) and insulin/oxaloacetic acid (Sigma). Lymphoid organs were minced using a 3ml syringe plunger and cells were put through 40u sieves. Cells were washed in media; red blood cells were lysed using an Ammonium-Tris-HCl (0.16M NH4Cl, 0.17M Tris, pH 7.2) solution. Cells were incubated in 2-5mls of solutions for 2.5minutes at 37C and then washed in media. Aliquots were made and stained with a 1:10 dilution of 0.4% trypan blue (Sigma) to count viable cells. Dilutions for the assays were taken.

Time course experiments: Spleen cells were plated at 2 x 10^6/ml, 10mls per sample for each time point. For all of the experiments described, spleen cells were used; T and B cells were not separated from each sample. Cells were stimulated with Concanavalin A (ConA) at 2.5ug/ml (Sigma). Nonstimulated samples were run concurrently. For all time points, samples from non-transgenic littermates were used as controls. Time points are 0, 15, 30, 60min, and 4hrs. At the given time point, cells are washed 2X with PBS and then lysed in 50ul of RIPA buffer with protease inhibitors (AEBSF, apoprotinin and Na orthovanadate). Samples are incubated on ice for 10min and frozen at -70^{0}C or used immediately to quantitate the total protein by the bicinchoninic assay (BCA), developed by Pierce (Smith, 1985). For all transduction experiments, protein levels have been quantitated using the BCA assay.

For in gel assays using myelin basic protein (MBP), 30ug protein lysate is used at a concentration of 0.5mg/ml of MBP is used in the 10% SDS-PAGE gel. The gels are washed, denatured and then incubated 1hr at room temperature (RT) in kinase buffer with 40mM ATP and ^{32}Pgamma ATP. They are then washed for approximately 2hrs. or until the background is significantly decreased, (as determined by using the geiger counter and comparing the intensity of radioisotope around the edges to the lanes), in 1% pyrophosphate and 5%TCA, stained with Coomassie blue, destained (50% methanol,

40% H_20, 10% acetic acid) and then exposed for a few hours on the phosophoimager and then left down on film for 3-5days (-70C).

For phosphotyrosine assays, 40ug of protein lysate is run on a 10% SDS-PAGE gel. The gels are transferred onto Immobilon-P (Millipore) filter paper. The filters are presoaked in Tris-glycine buffer (1.5gms Tris, 7.2gms glycine, 100mls methanol, qs500mls H_20, pH8.3) and then transferred in transfer buffer for 1hr at 75mA. Filters are then incubated with a monoclonal mouse IgG phosphotyrosine antibody (Santa Cruz) in assay buffer (1ug/ml) overnight or 1hr at room temperature (RT). The antibody is very specific and does not cross react with phosphoserine or phosphothreonine. The filters are then washed and incubated for 4-6hrs. with the secondary HRP-conjugated anti-mouse antibody at 4^0C. Samples are then developed using chemoluminescence (Amersham); equal volumes of detection agents 1 and 2 are added to the blots, which are incubated for 1min. The filters are drained and the blots are exposed on film or the phosphoimager. Chemoluminescence is a method used in which HRP catalyzes the oxidation of luminol which is excited. As it decays, it emits light which can be detected by autoradiography. Amersham includes an enhancer to amplify the amount of light emitted (ECL system). Blue light sensitive autoradiography film (Hyperfilm ECL, Amersham) is used and filters are incubated for 5sec. to 5min. depending upon the intensity of staining. (Rosewell, 1978; Cory, 1996)

For immunoprecipitations, protein lysate samples (40-50ug) are precleared. They are incubated with 5ug rabbit immunoglobulin in a volume of 50ul for 30min at 4^0C which will decrease background by binding non-specific immunoglobulin. The cleared samples, 40-50ug of protein lysate, are incubated with the given antibody (i.e. Grb-2 with 2ug/sample, Santa Cruz). Samples are then run on the 10% SDS-PAGE gel; the gels are transferred and blocked. Filters are incubated with the phosphotyrosine antibody and then developed as described above.

Chapter 4: Results

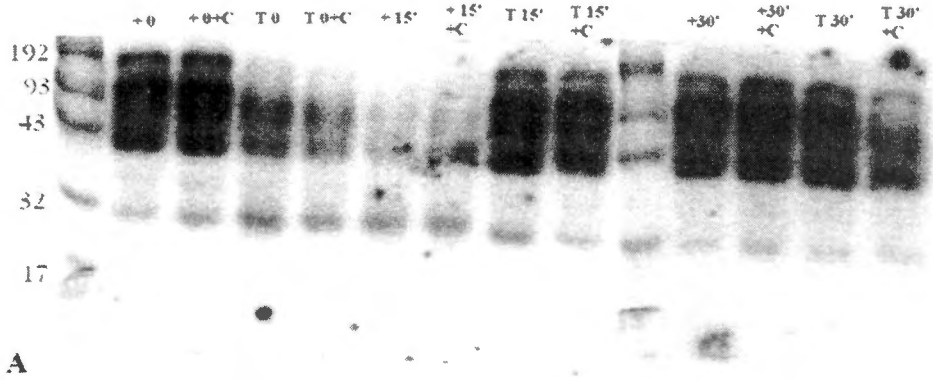
For all assays, cells are rested overnight before the assay is performed. Equal amounts of protein, as determined by bicinchoninic assay (BCA), are used for each assay. Coomassie blue staining of the gels is used to help confirm equal amounts of protein per lane/sample. All assays have been repeated on samples from both transgenic lines (Tg5550 and Tg5562) and the results described have been confirmed/repeated. Figure 4-1A illustrates the in gel kinase assay using MBP as a substrate. This assay is used to assess whether there are kinases present within the sample which can phosphorylate MBP as a substrate. The samples are separated out by molecular weight and the MBP is cross-linked/fixed in the gel. When ^{32}P is added, any kinases present will phosphorylate the MBP, and one will detect the band of the kinase which has phosphorylated the MBP. The MBP is fixed within the gel, and therefore the size of the band detected is a reflection of the size of the kinase only. Depending on the nature of the sample and the number of kinases present within the sample, there may be one or multiple kinases present; accordingly, one may get one or several bands on the

Figure 4-1: Cell signaling assays.

A. Autoradiograph of Myelin basic protein (MBP) assay comparing nonstimulated and stimulated spleen samples from wild-type and transgenic animals over a time course. Transgenic samples are labeled as "T"; Con A stimulated samples are labeled as "+C". 0=time 0min.; 15'=15min, 30' = 30min. Protein standards are in lanes 1 and 10. Transgenic samples are in lanes 4,5,8,9,13 and 14. Wild-type samples are in lanes 2,3,6,7,11 and 12. Nonstimulated samples are followed by stimulated samples.

B. Phosphotyrosine assay on wild-type and transgenic spleen samples from nonstimulated and stimulated samples over a time course. Transgenic samples are labeled as "T". Con A stimulated samples are noted as "+C". Time points are 0=omin, 15'min, 30'min,and 1hr. Wild-type samples are in lanes 1,2,5,6, 9,10 and 15. Transgenic samples are in lanes 3,4,7,8, 11,12,13 and 14.

C. Assay of unprecleared samples immunoprecipitated with Grb-2 and probed with an antiphosphotyrosine antibody for wild-type and transgenic spleen samples. Time course was performed with stimulated and nonstimulated samples. Transgenic samples are labeled as "T". Con A stimulated samples are denoted by "+C". Time points are 0min, 15min, 30min and 1hr. Transgenic samples are in lanes 1-8, and wild-type samples are in lanes 9-16.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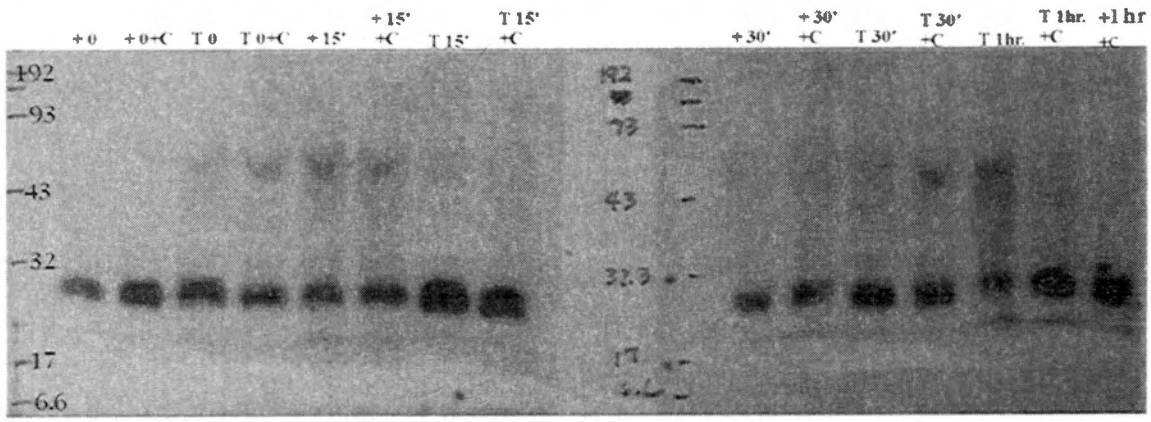
A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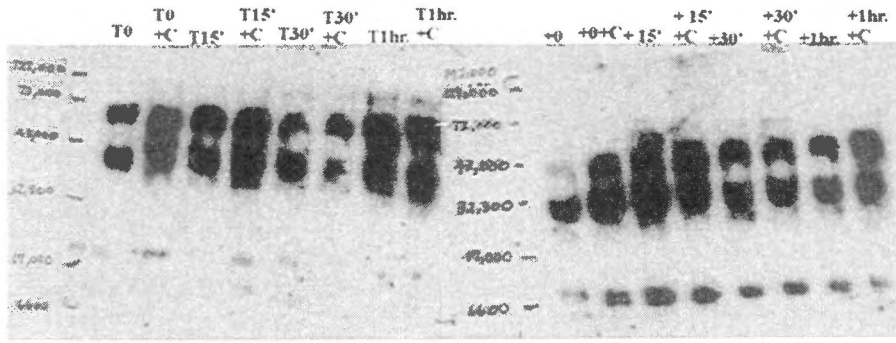
B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+1hr +C

C

autoradiograph. There are several proteins which are phosphorylated in the resting normal cells that are not phosphorylated in the resting transgenic cells. At 0hr, in the wild-type cells there is heavy phosphorylation of a band approximately 192kd and one band 127-192kd which are not present in the transgenic resting cells. Additionally, there is higher phosphorylation of a band about 93kd. In the 0hr transgenic samples, besides lacking the higher phosphorylated bands, there is a band about 37kd which is more intensely phosphorylated in the transgenics. For both the transgenic and wild-type cells there are no differences in phosphorylation between the stimulated and non-stimulated samples.

At 15min, the wild-type cells have very little if any phosphorylation of the 192kd band, the band between 127-192kd as well as the doublets between 73-127kd. In contrast, all of these bands are heavily phosphorylated in the transgenic animals. The phosphorylation pattern of the 15min samples from the transgenics appear like the 0hr wild-type samples. The only exception is that the low 37-43kd band appears to be phosphorylated a little more intensely in the stimulated transgenic sample vs. nonstimulated. There are no other differences in phosphorylation patterns between stimulated and nonstimulated samples at 15min, suggesting that these differences are independent of Con A stimulation.

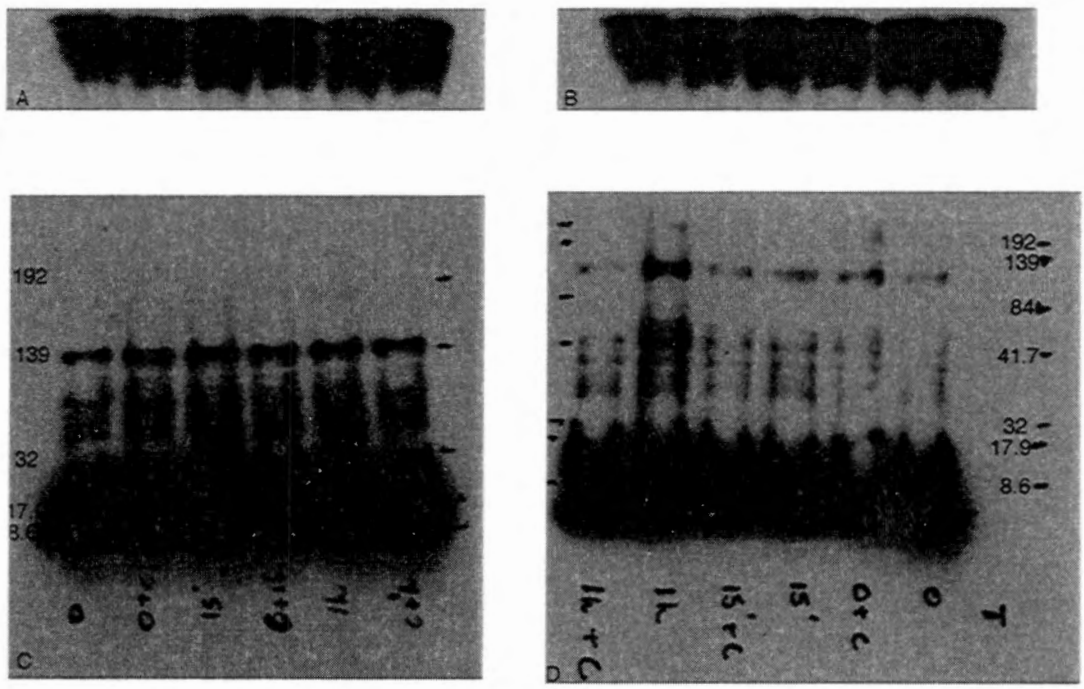
At 30min, the phosphorylation pattern of the wildtype animals is very similar to the 0hr wild-type and 15min transgenic samples. There are no apparent differences in phosphorylation between stimulated and nonstimulated samples. At 30min, in the transgenic samples, there is differential phosphorylation of the 192kd band between stimulated and nonstimulated samples. There is increased phosphorylation of this band in the stimulated sample. In addition, in both the wild-type and transgenic samples there is decreased phosphorylation activity of the low 37-43kd band.

In order to further investigate the (downstream) signaling differences between WASP transgenic animals and wild-type animals, phosphotyrosine assays were done. These assays identify proteins in the sample which contain phosphotyrosine residues. They are specific for phosphotyrosines and not serines or threonines. It often allows the identification of potential tyrosine kinases because many of these kinases have phosphotyrosine containing residues. Figure 4-1B demonstrates that in the resting state (cells are rested overnight before being stimulated), there is a baseline phosphorylation of a 40-50kd protein in the transgenic vs. wild-type animal. This is phosphorylated by 15min in the wildtype animal whereas levels decrease in the transgenic cells. At 30min, there is a very low level of phosphorylation in the wild-type, but higher levels of phosphorylation in the transgenic animal. At 1hr, it is more heavily phosphorylated in

the wild-type vs. transgenic cells. Basically, phosphorylation of this protein is opposite in the transgenic vs. wild-type cells starting at time 0min, when it is already phosphorylated in the transgenic cells. This assay has been repeated using samples from both transgenic lines, and the differences in phosphorylation were consistent. There are several putative proteins which are 40-55kd, including the ERKs and SHCs. The ERKs are signaling proteins involved in both T and B cell activation. SHCs are phosphoproteins involved early in the cascade of T cell signaling. Follow up studies will include immunoprecipitations of these proteins to identify this/these protein(s).

In the phosphotyrosine assays, Figure 4-1B, there is also a low molecular weight protein which is heavily phosphorylated in all samples. Based on the molecular weight of this protein and the proven association of Grb2 with WASP in the signal transduction cascade, immunoprecipitations with Grb-2 followed by screening with phosphotyrosine were performed. Figure 4-1C is an unprecleared sample and Figure 4-2 are the precleared samples. Normally samples are precleared to remove nonspecific immunoglobulin which will possibly decrease the number of nonspecific bands. One time the samples were not precleared and the assay revealed the results in Figure 4-1C. When the precleared samples were run (Figure 4-2), one would expect to see a decreased number of bands compared to nonprecleared samples but not differences in levels of

Figure 4-2: Grb-2 immunoprecipitation followed by probing with antiphosphotyrosine antibody over a time course. Molecular weight standards are included. Transgenic samples are labeled as "T". Time course is 0 min, 15min and 1hr. Con A stimulated samples are labeled as "+C".
A) +/+ 5sec. exposure
B)Transgenic sample 5sec. exposure
C) +/+ 1min. exposure
D)Transgenic samples 1min. exposure



Wild-type

Transgenic

phosphorylation or decrease in the number of bands in the nonprecleared samples. The unprecleared sample Figure 4-1C (and the precleared samples, Figure 4-2) demonstrate(s) the markedly reduced presence of a very low molecular weight band between 6.6 and 20kd, suggesting that this protein is hypophosphorylated in the transgenics. These studies are being repeated to determine if these differences are consistent. In examining Figure 4-2A and B, at 5 sec. in 2A , in the wild-type samples, there are two bands present which are 8.6-17.9kd compared to the transgenic samples which only has the higher molecular weight band phosphorylated. At 5min (Figure 4-2C and D), the samples are overexposed and it is difficult to discern the bands. This is the most significant finding of the immunoprecipitations. The other major finding is that in the transgenic samples there is a consistently decreased level of phosphorylation if all bands present compared to the wild-type. For all the samples, protein quantitation was done and an equal amount of protein was loaded per well. Therefore, this represents a decreased level of phosphorylation in all transgenic versus wildtype samples, regardless of the time point and whether the sample was stimulated.. The findings in the nonprecleared samples (Figure 4-1C) are real, and suggest that there is a low molecular weight protein which is capable of binding immunoglobulin, and is therefore removed when samples are precleared, but it is differentially phosphorylated in the transgenic

compared to the wild-type samples. Therefore we want to repeat these results 1) to prove the results are consistent between samples tested 2) to determine if the results are consistent between precleared and non precleared samples. If these results are consistent, it supports that there is a protein which can bind to immunoglobulin which is hypophosphorylated in the transgenic versus the wild-type animal. If so, possible proteins in the signal cascade need to be identified and follow up immunoprecipitations should be conducted to determine the identity of these proteins and their association with WASP.

Chapter 5: Discussion

In order to address where and how WASP affects the cell's regulatory events, several signaling experiments have been done to determine/confirm whether WASP affects the cell signal transduction cascade by differential phosphorylation of various proteins and/or by altering activity of some kinases. Based on the differential phosphorylation of these proteins, future experiments will be designed to identify what proteins are involved, to localize WASP in the signaling cascade, and to determine the function of WASP based on its role in the signaling pathways. Previous studies have demonstrated that WASP interacts with signaling molecules, cdc42Hs, nck, rho and more recently with the kinases, *fyn* and *Itk*. In addition, WASP has been associated with a role in actin polymerization, in that overexpression of WASP *in vitro,* alters actin polymerization. Therefore, we believe that overexpression of WASP *in vivo* would alter the signal transduction cascade, and this would result in changes in phosphorylation patterns of those associated proteins. Based on this hypothesis, we designed some initial experiments (1) to determine if there were proteins that were differentially phosphorylated between transgenic and non-transgenic cell cultures and (2) to see if these patterns changed over time.

At this time we have identified several kinases which differentially phosphorylate MBP in transgenic vs. nontransgenic bulk spleen cell cultures. In the MBP gel kinase assays (Figure 4-1A), the transgenic and wild-type samples have a temporally different MBP phosphorylation pattern. There is no differential pattern between stimulated and nonstimulated samples until 30min, suggesting that these differences are independent of Con A stimulation. There are differences at time 0hr, even after the cells have been rested overnight, which suggests that these differences are intrinsic to the expression of the transgene. This is further supported by the fact that these results have been repeated with samples from the other transgenic line (Tg5550). Most likely, these kinases are downstream of WASP, and in order to try to localize WASP within the cascade and to try to more logistically approach the identification of these proteins(kinases), several phosphotyrosine assays were conducted. Again, Con A was used as the mitogen. In the phosphotyrosine experiments (Figure 4-1B), again the phosphorylation pattern between the transgenics and nontransgenics appears to be inverted/reversed at different time points, and the predominant bands involved are between 43 and 73kd, which is similar in size and pattern to the ERK doublet. Because of the role of ERKs in cell signaling, proliferation and apoptosis, this makes the ERKs good candidate proteins for future immunoprecipitations to determine whether they are involved in WASP signaling. It is possible that overexpression of WASP affects the ERKs because it has been

demonstrated that B cell proliferation can involve the CD40 and ERK pathways. Sutherland et al (1996) demonstrated that in order to get T cell dependent B cell proliferation through CD40/CD40L, the ERK, JNK and p38 pathways are activated. If only the ERK pathway is activated, cells undergo apoptosis. Other support for the involvement of the JNK pathway comes from Zhang and Lamarche. They have demonstrated that cdc42hs interacts with rac which can then activate either a PAK 65/JNK/MAP/p38 pathway or p160ROCK pathway which affects actin organization and lamellopodia. Figure 4-3 illustrates the association between CD40, ERKs and proliferation. Figure 4-4 illustrates the association between cdc42, PAK65 and proposed location of WASP in the signal pathway. It should be kept in mind that these assays have been done on spleen cultures that contain B and T cells and thus one could see the affects of B cell stimulation present. Teleologically, it would follow that WASP somehow activates the ERK and CD40 pathways; this causes the B cell hyperproliferation, that results in the marked germinal center hyperplasia exhibited by these transgenics. In addition, depending upon the stimulus, or if there is an alteration in the signaling pathways, temporally, or depending upon the nature or presence of a(n) stimulus/antigen being present, the ERK pathway may only be activated and the cells

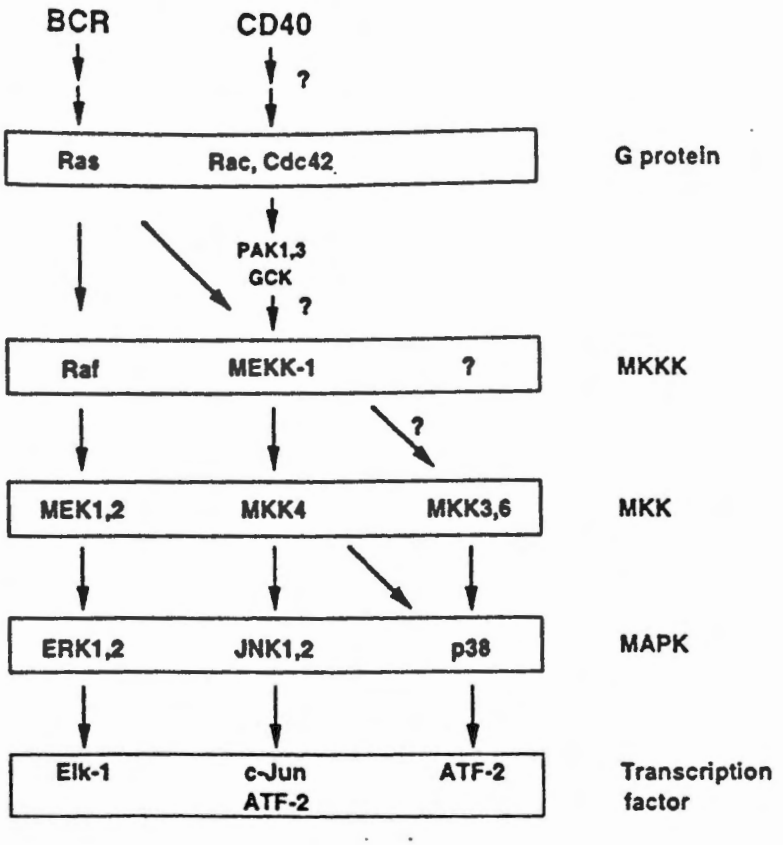
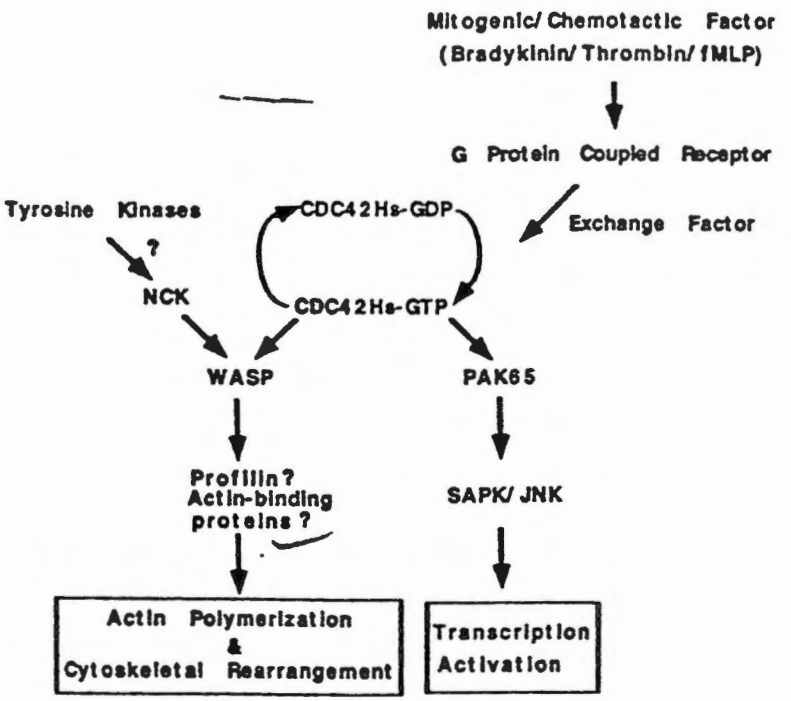


Figure 4-3: Cell signaling pathways involving CD40/40L and ERKs. Proposed scheme for the regulation of MAP kinases by the BCR and CD40 (Sutherland, 1996).



In this model, CDC42Hs plays a pivotal role in delivering signals both to the nucleus via PAK65 and a MAP kinase cascade and to the cellular cytoskeleton via WASP. WASP may be targeted to particular cellular locations, possibly via its C-terminus, and then may recruit proteins such as profilin to nucleate new actin polymerization. There is also evidence that WASP may receive signals from tyrosine kinases via the adaptor protein NCK (Rivero-Lezcano et al., 1995). The upstream regulation of CDC42Hs may involve G protein–coupled receptors such as the bradykinin receptor (Kozma et al., 1995). In neutrophils, chemotactic receptors may feed into the CDC42Hs–WASP pathway, thereby producing the cytoarchitectural rearrangements necessary for cellular movement.

Figure 4-4: Cell signaling pathways illustrating the possible association between cdc42hs, PAK and WASP (Symons, 1996).

may undergo apoptosis. In the WASP transgenic mice, the B cells are upregulated, as evidenced by increased expression of CD40, B71 and B72. However in the T cells, CD28 is upregulated, but not CD40L. Perphaps there is a defect in the signaling at this level, which alters the CD40 pathway such that only the ERKs are stimulated and that is why there is also a considerable amount of apoptotic cells in the germinal centers as well.

Finally, in order to start to identify proteins which are differentially phosphorylated in transgenic and non-transgenic lymphocytes, initial immunoprecipitations were done. Based on the proven association between WASP and Grb-2 and based on the molecular weight of the low band on the phosphotyrosine assays, immunoprecipitations followed by a phosphotyrosine assays were done to determine if Grb-2 was in fact differentially phosphorylated. From the unprecleared sample (Figure 4-1C), it certainly appears as though there are differences in Grb-2 or proteins associated with Grb-2. Because it is an immunoprecipitation, when the antibody to Grb-2 is added, it will precipitate out not only Grb-2, but any proteins bound, or tightly associated with Grb-2. In this case, there is a very low molecular weight protein (6.6-17kd) which is present in the wild-type animals but not the transgenics. If this is not Grb-2, another likely candidate would be the zeta chain of the TCR which is known to help regulate activation and anergic responses. The highest molecular weight band is the heavy immunoglobulin chain (50-

55kd). In both the precleared (Figure 4-2) and the unprecleared (Figure 4-1C) sample, the low molecular weight band (6.6-17kd) is present in the wild-type but in the transgenics, it is present at very low levels in the unprecleared sample and not present in the 5sec. exposure of the precleared sample. This finding represents the most significant results of this assay. Either Grb-2, or a Grb-2 associated protein is markedly hypophosphorylated in the transgenic samples, regardless of the time point. This suggests that this hypophosphorylation, may be due to the constitutive overexpression of the transgene.

One possible explanation for the differences in protein phosphorylation between the transgenics and non-transgenic cells is that at time zero, the wild-type cells are not activated and they may have inhibitors which are present, or have molecules which are already phosphorylated in the inactive state. In the transgenics, where cells may be constitutively activated at a low level from WASP and further downstream, the inhibitors may not be present, allowing the pathway from WASP and downstream, to be active at time zero. Subsequently, after initiating the experiment the normal chain of events for the wild-type cells causes the rapid dephosphorylation of some proteins (15min) which stimulates downstream events and eventually cell proliferation, apoptosis, etc. These proteins which are heavily phosphorylated at time zero, are only involved in

the initial phosphorylation events. Once they initiate downstream signaling, their job is complete and because they are not involved in any further downstream events, nor is it desired to continue to stimulate the initial pathway, these proteins and signaling molecules should be "turned off". For the transgenics, they are already stimulated endogenously from the point of WASP and further downstream. So, at time zero, the "initiating proteins" are already dephosphorylated/ "activated". However, with further "artifical" stimulation, this initiates signaling to "turn off"/phosphorylate the initiating proteins. This stimulus is sufficient to override WASP's effects. As a result, the initiating proteins are "turned off" by phosphorylating them. Another possibility is that this is artifact, in that the differences seen between the transgenic and wild-type cells are accurate, but somehow taking the plates in and out of the incubator artificially and differentially affects the cells (i.e. heat shock proteins are activated). However, this is an unlikely possibility because the results are repeatable between samples from both transgenic lines and from samples from the same line collected during separate experiments. independent of Con A; Another argument against this being artifact is the phosphorylation pattern in the phosphotyrosine assay. In this assay (Figure 4-1B), which is also run on samples collected on a separate day, one sees consistent differences in phosphorylation pattern between the phosphotyrosine assay and the MBP assay (4-1A).

The amount of phosphorylation of the 43-73kd band is opposite between the transgenic and wild-type samples. At time zero, it is not phosphorylated in the wild-type, but it is in the transgenic. At 15min. it is phosphorylated in the wild-type, and it is decreased in the transgenics. Again, this has been repeated both with animals from the same line as well as the other line. [A final consideration is that these assays are on bulk cultures and therefore contain B and T cells and the phosphorylation events may be reflective of B and T cell activity. Although technically challenging, in the future signal experiments may need to be done on separate populations of T and B cells in order to differentiated T cell vs. B cell signaling events.

In the phosphotyrosine assay (Figure 4-1B), one sees phosphorylation of a 43-73kd band when there is decreased phosphorylation of bands on the MBP assay, except at 30min, when bands from the MBP assay are highly phosphorylated in both transgenic and to lesser degree in wildtype and then the band on the phosphotyrosine assay is still present. Based on these results, one theory/hypothesis which is consistent is that the early events of the phosphotyrosine and MBP assays involve phosphorylation of proteins which inhibit activation. Then in order to activate those cells, those proteins are dephosphorylated, and they are then active/functional. Their role is rapid and transient and then their function is done, so these bands are once again phosphorylated. Other

proteins are responsible for initiating and maintaining downstream events. In the phosphotyrosine assay the band which is phosphorylated is active. In the transgenics at 30min and 1hr, there also appears to be a lower molecular weight band (17-30kd) which is phosphorylated at a very low level--which is not present or only at very faintly visible in the wild-type samples in the 30min and 1hr stimulated samples. If this top band is the immunoglobulin heavy chain, there still is differential expression of the lower band of 17-30kd. It is more heavily phosphorylated in the resting transgenic samples than wild-type. In the 15 min. transgenic samples, it is more heavily phosphorylated than in the 0min. In all transgenic samples it remains more heavily phosphorylated than in the other samples. It is thought that this lower molecular weight band could possibly be Grb-2 or the zeta chain of the TCR. This is also consistent because WASP has been directly associated with Grb-2 and because Grb-2 activity is essential for T cell activation. We proceeded to do immunoprecipitations with Grb-2 followed by incubation with phosphotyrosine antibody to see if Grb-2 was differentially phosphorylated. In the unprecleared sample, it appears as though the low molecular weight band (6.6-17kd) in the +/+ is much more heavily phosphorylated than in the transgenic. At resting, there is a very low level of phosphorylation and then after that, it is phosphorylated most intensely at 15min, and then decreases. In the transgenic samples, this band is hypophosphorylated,

which suggests that either Grb-2 or a molecule which immunoprecipitates with the Grb-2 antibody is markedly hypophosphorylated in the transgenic versus wild-type sample. The results from the precleared sample are consistent with this as well. In Figure 4-2A and B, in the wild type sample (A), two bands are heavily phosphorylated where as in the transgenic sample (B), there is only one band. The other band, the top band of the two bands as seen in Figure 4-2A, approximately 8-17kd, is not present. Thus, in both the precleared and unprecleared immunoprecipitations of Grb-2 probed with phosphotyrosine antibody, to assess differential phosphorylation of Grb-2, there is a band in the transgenic samples (8-17kd) which is markedly hypophosphorylated compared to the wild type sample. Therefore, either Grb-2 or a molecule in the Grb-2 complex is markedly hypophosphorylated in the transgenic samples regardless of the timepoint; this suggests that these changes may be due to constitutive overexpression of the transgene. Again, it does not appear to be Con A dependent, but these results are consistent. This suggests that the pathway affected is independent of Con A. Experiments should be repeated using anti-CD3, PMA/Ca and/or CD43 to determine if cells stimulated by these mitogens involve additional differentially phosphorylated proteins. If the hypophosphorylation of this band is due to constitutive overexpression of the transgene, then one would expect to see this decreased level of phosphorylation regardless of the

mitogen proved. Therefore, the experiments described above could provide additional data to support that overexpression of WASP results in the constitutive decreased phosphorylation of Grb-2, or a Grb-2 associated molecule.

How might the changes in phosphorylation represent changes in signaling that result in the differences in proliferation ? Why does one get changes in signaling? Through the use of these transgenics which overexpress the normal WASP protein, we have demonstrated that there is differential phosphorylation of several bands which are believed to be involved in the signal transduction cascade. At this time, these proteins have yet to be identified. Immunoprecipitations will have to be done to substantiate/determine which proteins are involved. One hypothesis is that WASP localizes early in the cascade and affects downstream events, one through rho, etc. which affects actin polymerization and cell morphology and a second pathway which affects signaling events through the ERKs/JNKs etc that then causes changes in transcription of IL-2 etc which lead to changes in cell proliferation. It is also possible that all these signaling events are one cascade/pathway. WASP could stimulate downstream events which do not divides, such that WASP may stimulate rho and the ERKs and JNKs all through one major pathway which affects actin polymerization and cell signaling. WASP may increase cdc42Hs and rho mediated events and these in turn affect phosphorylation

of Grb-2 directly, or WASP affects cdc42 which dissects downstream to stimulate some actin related events and also to stimulate TCR mediated events, such as JNKs and Grb-2, which affect proliferation and apoptosis.

Another hypothesis is that WASP's activity affects actin polymerization and the resultant changes in signaling alter cell proliferation secondary to the changes in cell morphology. The primary effects would include alterations in aggregation and adhesive properties, including cell surface marker expression. As a result of changes in actin polymerization and conformational changes in cell morphology, this would affect cell surface ligands and their function. Cells may not be able to maintain appropriate contact physically or between ligands temporally and spatially in order to transduce the proper signals. Thus, as a secondary event, cell signaling is altered, which changes cell proliferation/apoptosis. Some of these possibilities will be addressed in a future series of experiments examining cell aggregation properties, actin polymerization studies and EM using different mitogens. These studies will correlate functional changes, blastogenesis and aggregation, with structural changes in morphology by EM and actin polymerization experiments.

In trying to correlate the known information about WASP's role in cell and signaling and morphology with the clinical immunology of WAS, it is known that most patients'

immune cells have decreased proliferative indices compared to normal individuals. Perphaps, altered expression of WASP affects the pathway near the level of Grb-2 which interferes with the cells ability to respond to mitogens. As a result, although the cell binds antigen/mitogen, there are changes in signaling events (possibly involving the CD40/CD40L pathway or ERKs and JNKs), such that the signals are not transduced and transcription is not initiated. Consequently, both the morphology of the cells is altered, and they also do not proliferate adequately.

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PART V: OVERALL SUMMARY

Chapter 1: Summary

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disease in which the hallmark clinical signs involve thrombocytopenia, eczema and progressive cell mediated and humoral immunodeficiencies. Patients who survive through bone marrow transplants, splenectomies and supportive therapy are markedly at risk for the development of tumors and other autoimmune diseases such as arthritis and autoimmune hemolytic anemia (AIHA). The core defect of this disease is not known, nor is it understood why patients are markedly predisposed to neoplastic diseases. It is thought to be a stem cell mediated disease, and it is now known to affect cells from multiple lineages including platelets and lymphocytes. Its function in PMNs and monocytes is even more nebulous.

In order to ascertain the function of the WASP protein, transgenic animals were generated which overexpress the normal Wiskott-Aldrich syndrome protein (WASP) under a T cell promoter. This has allowed us to use these "gain of function" animals to elucidate the cellular and molecular basis for the immunopathology associated with overexpression of WASP protein. Several lines of these mice were generated which transmitted the gene in germline fashion. Expression studies were done by RT-PCR to

determine the levels and pattern of expression and to identify specific lines on which to focus. Two lines, Tg5550 and Tg5562, were selected based on their high levels of expression, the lymphoid specific expression, and severity of phenotype.

WASP transgenic mice, of lines Tg5550 and Tg5562, develop splenomegaly and a bilaterally symmetric lymphadenopathy by approximately 6-9 months of age. Histologically, both spleens and lymph nodes have marked germinal center hyperplasia with a medullary plasmacytosis. Hematology reveals depressed white blood cell counts, with a pancytopenia except for platelets. These animals have a mild thrombocytosis compared to their non-transgenic littermates. They have a corresponding megakaryocytosis of the spleens and bone marrow, with the bone marrows being otherwise unremarkable.

Based on histology, these animals appear to have "activated immune systems" in that they have marked and persistent germinal centers. In order to characterize the nature of these animals, several *in vitro* and flow cytometry experiments were done. Flow cytometry confirmed that there were increased numbers of B and T cells present in the spleens and lymph nodes of these animals. In addition, there were increased percentages of activated cells and/or upregulated expression of cell surface markers involved in cell activation. On B cells, there was an increase in percentages of CD40+ cells, as well as

increased numbers of B7-1+, B7-2+ and B7-1+/B7-2+ double positive cells. On T cells there was upregulated expression of CD28 and increased numbers of CD28+ cells, but surprisingly CD40L was not increased either in number of cells or amount per cells. Cells must be activated for some period of time (Roy, 1993) to see increases in CD40L expression. If the immune system overall is upregulated, it is expected that CD40L should also be upregulated. It may be necessary to perform *in vitro* stimulation experiments to determine if when cells are stimulated *in vitro*, transgenic cells are capable of expressing CD40L similar to the wildtype cells. If not, this suggests a defect in the CD40/CD40L pathway. If the transgenic cells do express CD40L when activated *in vitro* then there may still be a defect in the CD40/40L pathway of these transgenic cells or there may be insufficient stimulation *in vivo* to upregulate CD40L expression.

In addition, both splenocytes and peripheral lymph node samples had increased numbers of CD45RB+ cells. In the spleen, there were increased numbers of CD45RB+dull cells where as in the lymph node there were increased numbers of CD45RB+ bright cells. This suggests an increase in effector cell population, versus an increase in naive cells. If there were an increase in CD45RB+/mel-14+ cells versus mel-14+ cells one would expect increased numbers of T cells migrating through the sinusoids and a more predominant T cell hyperplasia. If there were an increase in CD44+ cells,

this would mean an increase in memory response. The increase in CD45+ cells suggests that the process (the activated immune response) is active and on going. The fact that there aren't increased numbers of memory cells despite the probable longevity of the "activated status" is interesting. Because almost all animals over 6 months that have been sacrificed have the increased number of germinal centers, the assumption is being made that this is an active process ---for some reason these animals are continually developing new germinal centers or maintaining the old ones, even though germinal centers usually only last for 3 weeks. It is also possible that there is an alteration in the threshold for an immune response versus anergy or tolerance, such that transgenic animals develop immune responses including germinal centers to a much lower dose of antigen than wildtype animals. This suggests some dysfunction either in B cells and or T cell/B cell interactions more likely since the transgene is expressed only under a T cell promoter. In this sense, it is also possible that there are dysfunctional T cell/B cell interactions which cause more germinal centers to be produced or there may be some defect in apoptosis within the germinal centers (increased number apoptotic cells) or there may be a related defect in the ability to generate memory cells.

In order to assess whether there were T cell or B cell defects/alterations which could result in the marked germinal center hyperplasia, a few assays were conducted to look at

T cell and B cell function. Blastogenesis assays using both T and B cell mitogens were performed on thymus, spleen and lymph node cell cultures. Samples from WASP transgenic thymii, spleens and lymph nodes all proliferated more when stimulated with Con A, and spleen and lymph node samples stimulated with anti-CD3 were hyperproliferative compared to nontransgenic littermate controls. These results suggest that at least when stimulated *in vitro*, T cells from transgenic animals proliferate more than T cells from +/+ animals due to overexpression of the transgene. B cells were hyperproliferative as well when spleen and lymph node samples were stimulated with LPS. This suggests that at least once stimulated, B cells have the capacity to proliferate more than +/+ cells, independent of T cells. Perhaps then this corresponds to the marked germinal center hyperplasia exhibited by these WASP Tg animals. Something, either antigen or overexpression of the transgene, stimulates the cells and as a result, they are activated, they upregulate cell surface markers, and they proliferate more, forming increased numbers of germinal centers. However, there must be some alteration in the T cell/ B cell interaction, even if it is strictly due to a T cell defect, related to the expression of the transgene, that is abnormal because despite the activated phenotype etc, and the marked increase in germinal centers, WASP transgenic mice do not have marked increases in immunoglobulin levels. They have mildly increased IgG levels, but IgM and

IgA are relatively normal. For this number of germinal centers, one would expect either marked increases in IgG or because there isn't a significant increase in CD44+ memory cells, these germinal centers could potentially be primary responses, but then one would expect to see increases in IgA and IgM. Again, these results suggest that there may be some defect in the T cell/ B cell intereaction, which may be due to an underlying T cell dysfunction (i.e. the result of transgene expression); however, *in vivo* experiments need to be done to determine the nature of this defect. Follow up experiments will include *in vivo* challenges with rabbit Ig, which will measure T cell dependent B cell responses, as well as LPS, which will assess T cell independent B cell responses. One could also measure IL-5 production both *in vitro* and *in vivo* to assess B cell dependent stimulation of T cells. In addition, RNase protection assays will be done on immune cell populations to determine whether there is any leakiness of the promoter. If so, this may help to explain the B cell hyperresponsiveness as well as probably the megakaryocytosis.

In order to further examine T cell responses, cytokine assays were conducted. Supernatants from cells which were stimulated *in vitro* were analyzed for IL-2, IL-4 and IL-6 production to assess T cell response and to determine if there were any changes in Th1/Th2 phenotype. Cells from both spleens and lymph nodes produced increased levels of IL-2 compared to cells from the nontransgenic littermate controls. This correlates

with the hyperproliferative responses illustrated on blastogenesis results and it also demonstrates that at least when stimulated *in vitro,* WASP transgenic T cells are capable of proliferating and producing IL-2, and in fact are hyperresponsive.

So, the next question which needs to be addressed is what causes these cells to be hyperproliferative, at least when stimulated *in vitro*.? For this, we have begun several signaling assays to assess whether WASP alters the signal transduction cascade such that constitutive expression of WASP upregulates downstream signaling events and results in increased proliferation indices. As a result, once the cell is stimulated or once WASP is stimulated there is increased phosphorylation of downstream events and this results in increased IL-2 production and proliferation. It is also possible that WASP provides just one signal for cell proliferation such that despite constitutive upregulation of WASP and associated downstream events, the cell still needs an additional signal (i.e. antigen) in order to stimulate cell proliferation. In fact, protein lysates from stimulated and nonstimulated samples over a given time course were examined for their ability to phosphorylate other proteins and to examine whether there were differences in phosphorylation of other proteins. In gel kinase assays using MBP as a substrate revealed several proteins which are differentially phosphorylated between tg vs +/+ over a short time course. This represents several differences in kinases which are differentially

activated, based on their ability to phosphorylate the MBP. Phosphotyrosine assays revealed two proteins which appear to be differentially phosphorylated. Further studies are being done now to try to identify what these proteins are which may be involved in the WASP signaling pathways. At this point, it does appear that WASP alters signal transduction cascades and as a result this could alter cell proliferation and lead to hyperproliferation of cells involved. So, perhaps overexpression of WASP upregulates downstream signaling events which may stimulate molecules such as the ERKS/JNKs/p38 which then result in increased proliferation of the cells and it is this higher level of constitutive gene expression that allows for stimulated cells to become hyperproliferative which results in the phenotype exhibited.

WASP has also been identified with several other molecules in the signaling cascade which are involved with actin, such as cdc42Hs and rho. In the process of localizing WASP within the signal cascade, it has been immunoprecipitated with cdc42Hs and nck among others. In addition, Symons et al demonstrated that altered expression of WASP affects actin polymerization as well. Therefore, some of the ongoing studies are to elucidate the role of WASP with actin and cell morphology. It is known that cells from many WASP patients have blunted micovilli and that their platelets are small. It has also been demonstrated that in order to get appropriate cell activation and responses, the

immune cells must maintain contact between each other temporally and spatially. Alteration of these events, affects cells' abilities to respond, and this can result in immune dysfunction.

Of the many molecules associated with WASP, one cell surface marker which has been intimately associate with WASP is CD43. This is a leukosialin cell surface marker which is thought to be involved in cell activation, adhesion/repulsion properties as well as thymic selection. For many years, it has had a very controversial role in WAS. In some studies, cells from WAS patients have altered cell surface expression or altered carbohydrate moieties or altered CD43 mediated functional responses. More recently it has been demonstrated that altered expression of CD43 either developmentally or spatially can alter cell adhesion, aggregation and immune responses. From all of this information, one can put together that if WASP function is altered it may cause changes in cell signaling which may lead to changes in actin polymerization which may directly or indirectly cause changes in immune function, possibly through CD43.

In order to address some of these issues, we first started with flow cytometry analysis of the resting form (115kd) of CD43. In these transgenic animals, it appears that there are decreased numbers of CD43 dull cells in the thymus. By comparison, there are increased numbers of CD43 dull and bright cells in the spleen and lymph nodes. It is not

clear what the significance of these findings are. The upregulation of the resting form of CD43 in the spleens and lymph nodes is associated with an activated response by the cells/host. The next steps are to 1) look at the different isoforms of CD43 to see if there are changes in the activated vs. resting isoforms in transgenics and non-transgenics. 2a) examine CD43 mediated proliferation via blastogenesis assays to determine if there are functional differences between transgenics and wildtype and 2b) to determine if the differences seen in WASP transgenics in general may be a factor of the differences in CD43 mediated proliferation 3) repeat signal transduction assays using CD43 as a mitogen to see if there is differential phosphorylation of proteins between transgenics and +/+ and to correlate these findings along with the findings from earlier signaling assays to determine if the differences in WASP transgenics can be attributed to CD43 mediated events 4) look at possible CD43 and/or WASP function through actin polymerization studies, EM analysis, and aggregation studies to determine the role of WASP in actin polymerization and to determine if there are any associations between WASP function and CD43. Using controls we will be able to assess the affects of just the WASP transgene on actin polymerization, and aggregation as well as then using different mitogens to see how this affects cells from transgenics and non-transgenic littermate controls. Future experiments also include obtaining or generating CD43 transgenic

animals to further analyze this relationship between CD43, actin polymerization, cell aggregation and WASP expression.

Obviously from these experiments, we have been able to demonstrate that the overexpression of WASP leads to a most interesting phenotype of an activated immune system. What is now even more challenging is to dissect the pathways of WASP signaling and WASP function.

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VITA

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She is presently pursuing a Large Animal Medicine residency at University of Tennessee College of Veterinary Medicine and continuing her research in Dr. Erby Wilkinson's laboratory.