To the Graduate Council:

I am submitting herewith a dissertation written by Ila Anne Davis entitled "An analysis of the Immunological Consequences of the Lack of Secondary Lymphoid Tissue: the Lymphotoxin-alpha Knock-out Mouse Model." 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 Immunology.

Barry T. Rouse, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

An Analysis of the Immunological Consequences of the Lack of Normal Secondary Lymphoid Tissue: the Lymphotoxin-alpha Knock-out Mouse Model

A Dissertation

presented for the

Doctor of Philosophy Degree,

The University of Tennessee,

Knoxville

Ila Anne Davis, DVM

August 1998

Dedication

This dissertation is dedicated first to the memories of my mother and father. The tragedies which so characterized their lives lead to their early spiritual, emotional, and physical deaths. But through their pain and suffering, they provided me the greatest gifts of all... the knowledge that I can take care of myself, the courage to pursue my goals regardless of obstacles, and the strength to work as hard as it takes to achieve satisfaction with myself.

Secondly, I dedicate this manuscript to by soul-mate, Eric Davis, for letting me take the wheel whether I needed to or not.

Finally, to the ever present guidance of my animal totems and companions from whom I am temporarily separated, in particular to Scooter, Moped, and Doc; I dedicate this work to you for the constant yet subtle reminders of what is good and gentle and necessary during my walk on this road.

Admissions of Gratitude

No words of thanks could possibly be adequate to recognize my committee members, both those official and unofficial, for the time and understanding they have given over the past several years inspite of the personal tragedies which tore through their own lives.

For you, Dr. Slauson, I hope there will always be peaches ready to eat.

For you, Dr. Wilkinson, I hope you'll always feel the warmth of the sun on your face, even when it's raining.

For you, Dr. Mucenski, I hope the family circle will never be broken.

For you, Dr. Bochsler, in addition to warmth, I hope you'll always have a Buddy and a bird in the bush to admire.

For you, Dr. Wetzel, I hope this new association will continue to blossom.

And finally, to my major professor, Dr. Rouse, for simultaneously inspiring both my curiosity and anger, for introducing me to a world which has unlimited possibilities, and for saving the praise for when it is truly deserved, I wish you continued success professionally. And personally, I thank you for your exuberant zest for life and for sharing Margaret, as she is truly your greatest asset.

Acknowledgements

Nothing great is ever achieved alone. The guidance and friendship of our former lab manager, Mr. Bill Wire, and our current lab manager, Ms. Teresa Sobhani, have made the past few years enjoyable and so very interesting. Several members of the Rouse lab have provided wisdom and guidance and friendship; most notably, Dr. Zhiya Yu, Dr. Siva Kanangat, and Shivaprakash Gangappa, Kathy Knight, and Dixie Dallas. Their willingness to share and to discuss have been invaluable. Dr. Buddy Moore and Dr. Dave Brian have both served as secret sources of light-hearted inspiration while Carol, Paula, Kim, Erin, and Geri have continuously served well above what was necessary. Diane Leslie has always provided an encouraging word and has managed, at times, to bring order to my chaos. Jim Wesley has been most accommodating with his knowledge and availability. Kreis, Phil, and Tommy at the CVM photo lab have been gracious with their wit and talent. Nancy Nielsen and Marilyn Kerley have been welcome sources of information. Dr. Sharon Witonsky taught me the five phases of being a graduate student, information which was invaluable at times. Finally, Sally Fridge, and the members of the lab animal facility (Andre, Peggy, Baily, and Jonas) have been helpful and have almost managed to make going to the mouse room fun. What a long strange trip it's been!

Abstract

Lymphotoxin-alpha (LT-α) is a non-antibody product of activated lymphocytes with functions very similar to those of tumor necrosis factor-alpha (TNF-α). Surprisingly, the genetic disruption of the murine LT-α gene attributed critical functions related to lymphoid tissue development to the protein product of this gene. Although characterized extensively with respect to receptor interaction and associated ligands, information regarding the role played by LT-α in immune responses are lacking. At the molecular level, LT-α is involved in key signaling events during embryogenesis which, when lacking, apparently cannot be compensated for post-partum. The cell types expressing LT-α in either its homotrimeric or heterotrimeric form during development remain undefined. Further, although the tissues which express the receptors for the LT-α complexes are presumed to be of stromal support tissue in origin during development, this also remains unsubstantiated. While the experiments described in this dissertation do not address these developmental conundrums nor do they explore the molecular events involved in lymphoid tissue development, they attempt to systematically define which components of the LT-α deficient mouse's immune system are impaired and which are normal.

Following the introductory information in Part 1, Part 2 explores which of the remaining lymphoid tissue compartments LT-α deficient mice are sufficient to provide immune responsiveness when isolated and used to reconstitute severe combined

immunodeficient mice. Next, in Part 3, the expression of specific vascular addressins and cell adhesion molecules are compared between wild-type and LT-α deficient mice. In Part 4, the unimpaired homing and functional ability of whole splenocytes from LT-α deficient mice is reconfirmed. Additionally, the inability of wild-type cells to correct the deficient phenotype following adoptive transfer is verified. Part 5 attempts to define the role played by the spleen in the generation of mucosal immune responses in LT-α deficient mice and confirms that these mice possess compensatory mechanisms for the maintenance, albeit at reduced levels, of immune function. Finally, Part 6 examines the ability of lymphocytes from LT-α deficient mice to recognize and respond, both humorally and in a cell mediated fashion, to a live replicating virus delivered enterally or parenterally.

The data presented in this dissertation is novel and as yet unpublished, the latter issue will hopefully soon be remedied.

Table of Contents

List of Figures

List of Tables

Part I: General Introduction and Overview

Chapter 1: Introduction to the TNF Family Players

Lymphotoxin-alpha (LT-α), a non-antibody substance released from lymphocytes, was initially described in 1967 in the context of its ability to kill syngeneic cells (1). Many functions of LT-α have been defined in the ensuing 30^{+} years, the majority focusing on its capabilities of inducing and promoting inflammation and cell death. The functions of LT-α closely mimic those of tumor necrosis factor-alpha (TNF-α). These lymphokines demonstrate over 35% amino acid homology (2-6), but because LT-α has a more restricted cellular source (activated lymphocytes only (7)) than TNF-α, LT-α has long been considered less potent and redundant to TNF-α. Both lymphokines map to murine chromosome 17 and are separated by only 1100 nucleotides within the H-2 major histocompatibility complex (MHC) (8,9). A very similar linkage is found in the human genome on chromosome 6 (10).

The close functional and structural homology of TNF-α and LT-α have resulted in their placement into a family of related inflammatory cytokines of which TNF-α is the prototype (11). The multiple activities of these two cytokines are mediated by two distinct cell surface receptors which are identified by their molecular masses as p55- and p75-TNF receptors (TNFR). Both TNF-α and LT-α interact as trimers with either p55TNFR or p75TNFR with seemingly equivalent affinity. Both receptors trimerize following appropriate ligand triggering (12). The intracytoplasmic domains of these receptors differ suggesting they are involved in distinct signaling cascades leading to unique events. Efforts to unravel the differential effects of triggering through these receptors began in the early 1990s and continue today. Generation of p55TNFR deficient

(-/-) mice by gene targeting leading to disruption of the coding sequence was initially reported in 1993. The loss of the p55TNFR resulted in no apparent physical or clinical abnormalities unless the mice were challenged with *Listeria monocytogenes*, a facultative intracellular bacteria (13, 14). Mice lacking the p55TNFR exhibited 100% mortality and severe debilitation in controlling bacterial replication and clearing bacteria from organs (14). Interestingly, these mutant mice were resistant to the lethal pathology induced by intravenous (iv) injection of TNF-α and to lethal dosages of bacterial lipopolysaccharide (LPS) from *Salmonella typhimurium* or enterotoxin B from *Staphylococcus aureus* (13, 14). Based on these reports, the p55TNFR was deemed crucial for host defense against certain microorganisms and their pathogenic factors.

Generation of the p75TNFR -/- mouse was described in 1994. This mouse was also generated by gene targeted homologous recombination and examined similarly to the p55TNFR -/- mouse. Not surprisingly, results supported different functional roles for these receptors but did not clearly define such roles. Specifically, in this system, p75TNFR-/- mice were less sensitive to the damaging effects of both subcutaneously and iv administered TNF-α than were wild-type (wt) mice. Further, although p75TNFR-/- mice showed increased mortality following a lethal dose of *L. monocytogenes* when compared to wt mice, the sensitivity of these mice to *Listeria* infection was much less than that of p55TNFR-/- mice (15). The fact that loss of neither receptor resulted in complete resistance to the effects of LPS indicated a type of cooperation between these receptors. "Ligand passing" was confirmed and helped further define the often over-lapping outcomes observed following signaling through the p55- and p75TNFRs (16).

Specifically, following LPS stimulation of effector cell populations, homotrimeric LT-α or TNF-α bind to the trimerized p75TNFR which serves to localize the ligand to the targeted cell's surface. The ligand is then released from the p75TNFR to adjacent p55TNFRs after which the intracellular signaling events leading to cell death or production of inflammatory mediators follow. Ligand passing, therefore, allows amplification of signals generated by TNF-α and LT-α. Obviously, other functions of these receptors cannot be explained by this model and await clarification.

The early descriptions of the mechanisms of action of homotrimeric LT-α and TNF-α at the TNFRs seemed to confirm the redundancy of LT-α as an inflammatory cytokine. Questions regarding the necessity of LT-α were frequent from the time of its initial description until early in 1994. Prior to this time, it was known that homotrimeric LT-α existed only as a soluble complex but surface bound LT-α was found associated with a 33 kilodalton (kd) integral membrane glycoprotein and was not in homotrimeric form. Browning, et. al., defined a type II transmembrane member of the TNF ligand family which associated as a dimer with a single molecule of LT-α, thus forming a surface complex (17). This anchoring molecule was called LT-β and the entire heterotrimeric complex was found expressed on the surfaces of activated lymphocytes, cytotoxic T cells (CTL), and interleukin (IL)-2 activated natural killer (NK) cells (18).

The LT-β genetic sequence was deduced from its amino acid sequence which was 21 and 24% homologous to the amino acid sequences of TNF-α and LT-α, respectively (17). The tandem arrangement of the TNF-α and LT-α genes was a clue that the LT-β

gene may be similarly localized. The LT-β gene was indeed found on the centromeric side of and just 2 kilobases (kb) from the TNF-α gene (17). **Figure 1** (page 17) illustrates the gene arrangements of LT-β, TNF-α and LT-α and represents both the human and murine chromosomal patterns.

The identification of the surface LT-α_1:LT-β_2 complex on activated lymphocytes and other effector cells led to speculation of a distinct role for LT-α independent of it's role as an inflammatory cytokine. This heterotrimer had no apparent interaction with either the p55- or p75TNFR although a minor form, LT-α_2:LT-β_1 could apparently bind to, but not trigger, both TNFRs (12, 17). Conversely, it was possible that LT-α had no functions independent from those of TNF-α and the role played by LT-β was solely to retain and thus inactivate LT-α. To confirm a specific role for the LT-α_1:LT-β_2 complex, a receptor needed to be found. Such a receptor was identified in 1994 by testing previously identified members of the TNFR family (19). One member, characterized in 1993, was known as TNFR–related protein ($TNFR_{RP}$). This receptor, because it neither bound nor neutralized toxicity mediated by TNF-α and LT-α but bound the two LT-α:LT-β complexes specifically and with high affinity, was renamed the LT-β receptor (LT-βR) (17).

A specific function for LT-α, independent of TNF-α and either of the TNFRs, was subsequently defined by the use of mice rendered deficient for LT-α, LT-β, and LT-βR, as discussed in subsequent chapters.

Chapter 2: Verification of a Specific Role for LT-α

Nearly simultaneous with the characterization of the LT-βR (17) came the initial description of the LT-α deficient mouse (20). Although not an embryonic lethal, disruption of the LT-α gene resulted in total ablation of secondary lymphoid tissues, including Peyer's patches (PP), and loss of splenic red and white pulp compartment delineation. This initial description was quickly followed by a second (21) in which several aspects of immune function and responsiveness in the mutant mice were investigated. Specifically:

1. splenocytes, peripheral blood lymphocytes, and thymocytes from mutant mice expressed comparable levels of L-selectin, the naïve lymphocyte homing molecule, as did their wild-type and heterozygous littermates;
2. bone marrow lymphocytes were equally capable of homing to and repopulating the empty lymphoid anlages of severe combined immune deficiency (*scid)* mice whether from mutant, wild-type, or heterozygous mice;
3. total serum IgM and IgG were similar in mutant and wild-type mice however serum and fecal total IgA levels were greatly reduced in mutant mice; and
4. mutant mice had severely impaired systemic IgG responsiveness to subcutaneously administered ultraviolet inactivated (uv) herpes simplex type I virus (HSV-I) and keyhole limpet hemocyanin (KLH).

In an effort to further define the role played by LT-α in the development of lymphoid tissues, Browning and colleagues headed up extensive research which produced a complete characterization of the murine LT-β gene (22). Although similarly located in

the MHC loci, the murine LT-β gene contained just 3 exons. The loss of a splicing site had apparently resulted in the joining of exons 2 and 3 and intron 2 into one large second exon. The human LT-β gene locus had 4 distinct exons with intervening intronic segments. The messenger ribonucleic acid (mRNA) of LT-β appeared to be constitutively expressed in the spleen, peripheral blood lymphocytes, thymus, and intestinal segments, perhaps reflecting its presence in gut-associated lymphoid tissues (22). Given that the expression of LT-α is induced, the appearance of surface associated LT-α_1:LT-β_2 appeared to be regulated by the factors which regulate LT-α (22, 23).

The actual role for LT-β in lymphoid tissue organogenesis was determined in 1997, again nearly simultaneously by two separate groups. The Flavell group (24) noted that LT-β deficient mice, when examined at 6-8 weeks of age, had disrupted splenic architecture, lacked Peyer's patches, mandibular, brachial, axillary, popliteal, inguinal, sacral and iliac lymph nodes but did possess mesenteric nodes and small cervical lymph node clusters. These mice had normal serum IgG and IgM levels but reduced serum and fecal IgA levels. The investigators speculated that the reduced IgA levels may have been due to the lack of Peyer's patches, despite the presence of mesenteric lymph nodes. Additionally, following intraperitoneal administration of sheep red blood cells, both LT-α and LT-β deficient mice generated higher antigen specific serum IgM but reduced IgG levels when compared to wild-type control mice. Interestingly, however, when the antigen used was nitrophenol-haptenated chicken gamma globulin, LT-α and LT-β deficient mice demonstrated isotype switching equivalent to that seen in wild-type mice but greatly impaired affinity maturation. Both mutant mice lacked splenic follicular

dendritic cells and follicle marginal zone macrophages, two components deemed essential for splenic germinal center B cell differentiation and affinity maturation (25).

The LT-β deficient mice generated by Rajewsky's group (26) were generated by the recently described *Cre-loxP*-mediated gene targeting method (27). This line of mutant mice had essentially the same phenotype as the Flavell mice with a few exceptions. Firstly, the Rajewsky LT-β deficient mice were maintained in a conventional facility while the Flavell mice were maintained under specific pathogen free conditions (24, 27). This, and the age at which mice were examined (Rajewsky, over 8 weeks of age; Flavell, 6-8 weeks of age) may explain the peritoneal fluid and peripheral blood B and T cell lymphocytosis observed in both the LT-α and LT-β deficient mice examined by the Rajewsky group. Additionally, the Rajewsky group noted marked perivascular accumulations of lymphocytes in lung and liver sections from both LT-α and LT-β deficient mice which were identified as being predominantly $B220^+$ and $CD4^+$ cells. In considering the observations presented in this dissertation, the age at which LT-α mice are examined and the conditions in which they are housed may greatly determine histologic findings on necropsy, viability in dirty environments, and immune responsiveness.

Based on the phenotype of the LT-β deficient mouse, LT-β, via it's interaction with LT-α, indeed plays a critical role in lymphoid organogenesis and in cellular distribution and compartment delineation within the spleen. Because both LT-α and LT-β deficient mice lack Peyer's patches, most peripheral lymph nodes, and splenic follicular dendritic cells and marginal zone macrophages, it is logical to accredit these cytokines

with these developmental functions. The presence of mesenteric and cervical lymph nodes in LT-β deficient but not LT-α deficient mice suggests a significant role for LT-α_3 in the development of specific lymphoid tissues. Interestingly, closer examination of p55TNFR-/- and double p55TNFR/p75TNFR deficient mice revealed poorly developed and reduced numbers of intestinal Peyer's patches (24, 28). Further, p55TNFR-/- mice lacked the ability to form primary and secondary follicular germinal centers, perhaps due to absent follicular dendritic cells (29-32). Therefore, LT-α_3 appears to play critical roles, through either its interaction with the p55TNFR or an as yet unidentified receptor, in the formation of Peyer's patches and lymphoid tissue germinal centers. Similarly, LT-α_1:LT-β_2 has a similar critical role, through its interaction with the LT-βR or an as yet unidentified receptor, in the formation of all peripheral lymph nodes except mesenteric and cervical nodes.

Because LT-α also interacts with the LT-βR, the summary of LT-α's functions are incomplete without mention of the effects of disruption of this association. Two separate reports of such disruptions appeared simultaneously in 1996 (33-34). The mice described by Ettinger, et. al., were transgenic for the expression of the murine LT-βR fused to a human Fc receptor (FcR) which had been mutated to inhibit both Fc binding and complement activation. The expression of this fusion protein (LT-βR-FcR) was driven by a cytomegalovirus (CMV) promoter and the protein was detectable in most tissues examined but only after Day 3 of life in transgenic pups (33). The LT-βR-FcR was expressed at variable levels in the transgenic offspring but in those mice with high transgene expression, body and splenic weights were reduced and PP were either reduced

in size and number or totally absent. All lymph nodes were present in all transgenic mice examined and appeared to be normal with respect to structure and cellular constitution. Splenic architecture was disrupted, however, with loss of marginal zones and disrupted B and T cell zones, reminiscent of both LT-α and LT-β deficient mice.

Using a similar approach to disrupt LT-βR triggering, Rennet, et. al., designed an LT-βR-Immunoglobulin IgG_1 (IgG_1) fusion protein (LT-βR-IgG_1) which was administered iv to timed pregnant female mice to achieve gestational blockade of the LT-βR (34). The advantage of this system over that of Ettinger's is the ability of LT-βR-IgG_1 to bind the murine Fc receptor (FcRn) and therefor cross the yolk sac. LT-βR-IgG_1 functioned as a soluble receptor decoy for LT-α_1:LT-β_2 thus preventing interaction of LT-α_1:LT-β_2 with the endogenous LT-βR. The LT-βR-IgG_1 fusion protein was injected on days 14 and 16 of gestation based on the reported times at which LT-βR mRNA had been detected in murine embryos. Offspring pups had no Peyer's patches, inguinal, or popliteal lymph nodes. Lymphatic and circulatory vessels were present but no stromal tissue anlages were detected for the missing lymphoid tissues. Surprisingly, brachial and mesenteric lymph nodes were present. The authors went on to determine the temporal ordering of lymph node development by injecting their LT-βR-IgG_1 fusion protein beginning at day 9 of gestation through day 18. Interestingly, mesenteric lymph nodes and spleen were present in all offspring. Brachial lymph nodes formed after day 12, axillary nodes after day 14, inguinal nodes after day 15, and popliteal nodes after day 16 of gestation. Splenic architecture was disrupted throughout with lack of B and T cell

compartmentalization, lack of follicular dendritic cells, and lack of germinal center formation.

Finally, the effects of the LT-βR-IgG_1 fusion protein on splenic organization were expanded by MacKay, et. al., to show that the development of germinal center components are critically dependent on LT-α_1:LT-β_2 signaling throughout the lives of adult mice (35).

The reports by Ettinger and Rennet (33, 34) provided further clarification of the role of LT-α in the formation of lymphoid tissues but a complete understanding remains elusive. Clearly, however, LT-α plays a critical role in the development of normal splenic architecture and in the formation of Peyer's patches. LT-α is clearly essential for the development of lymph nodes but must be expressed at very specific time points during gestation to achieve normal nodal development. Further, based on the work by Mackay (35), the effects of LT-α on lymphoid tissue organogenesis are developmentally fixed but LT-α is apparently required for the continued formation of normal splenic and lymph node follicular and germinal center components. The missing piece to the LT-α puzzle, then, appears to be the identification of the cell types which express either LT-α:LT-β complex or LT-α_3. Further, the trigger which induces ligand expression on such cells in the embryo, given that LT-α is an activation induced cytokine, awaits definition.

Chapter 3: A Brief Over-view of the Mucosal Immune System

A major branch of the immune system operates to protect the mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts from foreign antigens and microbial pathogens in the external environment. This branch, called mucosa-associated lymphoid tissue (MALT), protects the epithelial surfaces which cover the internal mucosa of all body cavities by providing protective secretory antibodies. The MALT is a highly compartmentalized, organized, and nearly autonomous branch of the systemic immune system. Anatomically speaking, the MALT is made up of multiple microdivisions which serve as primary mucosal inductive sites where the immune responses which result in protective antibody secretion are generated. Such microdivisions include the Peyer's patches of the small intestine, lymphoid follicles of the cecum and large intestine, and tonsilar tissues of the oropharynx.

The gastrointestinal tract possesses a unique portion of MALT known as gut-associated lymphoid tissues (GALT). Here, specialized follicle associated epithelial (FAE) cells, termed 'microfold' (M) cells, are highly efficient in the transcytosis of macromolecules, particles, and microorganisms from the luminal surface to underlying lymphoid tissues (37). M cells are concentrated over nodules of lymphoid tissues called Peyer's patches (PP) which serve as primary GALT inductive sites. Additionally, within the parenchyma of mucosal organs and glandular tissues, are large numbers of diffusely scattered lymphoid cells which establish the mucosal effector tissues. In the gut, lying between epithelial cells and adjacent to the basement membrane, are the intraepithelial lymphocytes (IEL) which are predominantly (90%) T lymphocytes of the $CD8^+$ subset

(37). The lamina propria (LP), which is separated from the epithelial cell layer by a thin basement membrane, contains most components of the immune system with B cells and plasma cells predominating (38). The IEL and LP compartments, therefore, represent the effector arm of gut mucosal immune responses and function to disseminate the generated immune responses throughout the length of the intestinal tract.

Specific to mucosal immune responses generated within the gut, luminally presented antigens have several possible fates following gut absorption. Antigen may be taken up either by intestinal epithelial cells, M cells, or pass to the mucosa via paracellular routes. Uptake by intestinal epithelial cells, which do express MHC class II molecules and can therefor act as antigen presenting cells (APC), is suspected by many investigators to lead to tolerance to the antigen, particularly if it is a soluble or inert protein (39, 40, 41). This route of entry seems to preferentially involve stimulation of $CD8^+$ IELs which in many situations have been shown to be antigen non-responsive (42, 43). The expansiveness of the intestinal epithelium and the location of the IEL may have evolved so that the multitude of antigens present in the GI tract would initially encounter T cells which were non-reactive and perhaps inhibitory so that local and systemic immune responses would be impeded. This provides good explanation for the relatively low incidence of food-related allergies in contrast to the large number of food-related antigens ingested (44, 45, 46).

Induction of active immune responses in the GI tract involving both humoral and cell mediated components is believed to require antigen entry through M cells to underlying components of the PP (47). In fact, several studies have shown that the

induction of IgA immune responses in the gut requires that antigen enter the nodular lymphoid tissues of the GALT at some stage of the immunization process (48, 49, 50). The small number of M cells when compared to the total number of enterocytes in the length of the intestinal tract suggests that their contribution to the generation of mucosal immune responses would be quite small. However, their position over PP and the organization of these highly specialized lymphoid nodules allows nearly exponential amplification of immune inductive events. Several microorganisms, including *Salmonella* and *Reovirus,* are known to selectively bind and gain access to the underlying GALT components through M cells (51). This selectivity is due to the physical character of the luminal surface of M cells, their relative accessibility when compared to enterocytes due to the thinner covering of glycocalyx, and the diversity of M cell surface oligosaccharides which are exploited by specific microorganisms as receptors (52, 53, 54). Invaginations of the basolateral surfaces of M cells house B and T lymphocytes and occasionally macrophages and dendritic cells (DC). Following transit through the M cell, antigen is passed either intact or in the context of MHC Class II to the intimately associated lymphocytes and professional APCs (52). Lymphocytes receiving appropriate antigenic stimulation pass into efferent lymphatic channels, located at the bases of the PP and throughout the LP, to the mesenteric lymph nodes (MLN) where they mature further. In the case of professional APCs, the antigen is received intact from the M cell, processed and presented either in MHC Class I or II restriction, and presented locally to PP T cells or to lymphocytes residing in the draining lymph node. A limited number of memory B cells, but very few plasma cells, reside within the folds of M cells (55). Receipt of

appropriate antigenic and costimulatory signals leads to their rapid activation and migration to locations within the LP where they may terminally differentiate to IgA secreting plasma cells.

Antigen entering the mucosa by paracellular routes are likely to initially contact resident macrophages or DCs. Either cell type is capable of antigen uptake and processing however controversy exists regarding both their type of antigen presentation and the outcome of antigen recognition. Either active immunity or tolerance may be generated depending on the degree of local inflammation and available costimulation (56, 57). These issues, as well as the migratory patterns of these LP APCs remain incompletely understood.

In addition to professional APC's, the LP contains T cells predominantly expressing the α/β TcR and of an approximate 2:1 $CD4^+$ to $CD8^+$ ratio (38). While these cells may be involved in the costimulatory functions leading to either the generation of local humoral immune responses or pathological processes, their cytokine profiles reflect no predetermined bias but their presence appears to be essential for the regulation and differentiation of LP IgA-secreting plasma cells (38, 58).

Figure 2 (page 18) serves to illustrate the anatomy of the various components which constitute the GALT and MALT.

The diverse population of immune cells within the GALT are capable of carrying out inductive and effector functions due to their migratory patterns which ensures the placement of responsive cells in optimal locations. Naïve T and B lymphocytes are capable of localizing to M cell invaginations within PPs because they express the naïve

lymphocyte homing molecule, L-selectin, which allows access to the high endothelial venule endothelium of PPs and peripheral lymph nodes (59). Following appropriate stimulation, activated lymphocytes travel within lymphatic vessels to the mesenteric lymph node where maturation and proliferation occurs. Additionally, L-selectin expression is down-regulated and molecules which permit re-entry to mucosal tissues are expressed on the activated cell's surface. From the MLN, the cells pass to the thoracic duct and then to the blood which transports them back to the LP. Access to the LP across venular endothelium, which expresses the vascular addressin MAdCAM-1, is achieved by ligation of the activated lymphocyte expressed homing molecule, $\alpha_4\beta_7$ (59). The addressin and homing molecules involved in populating GALT are discussed in greater detail in Part 3 of this manuscript.

Figure 3 (page 19 & 20) illustrates the possible recirculation patterns for the generation of IgA committed B cells.

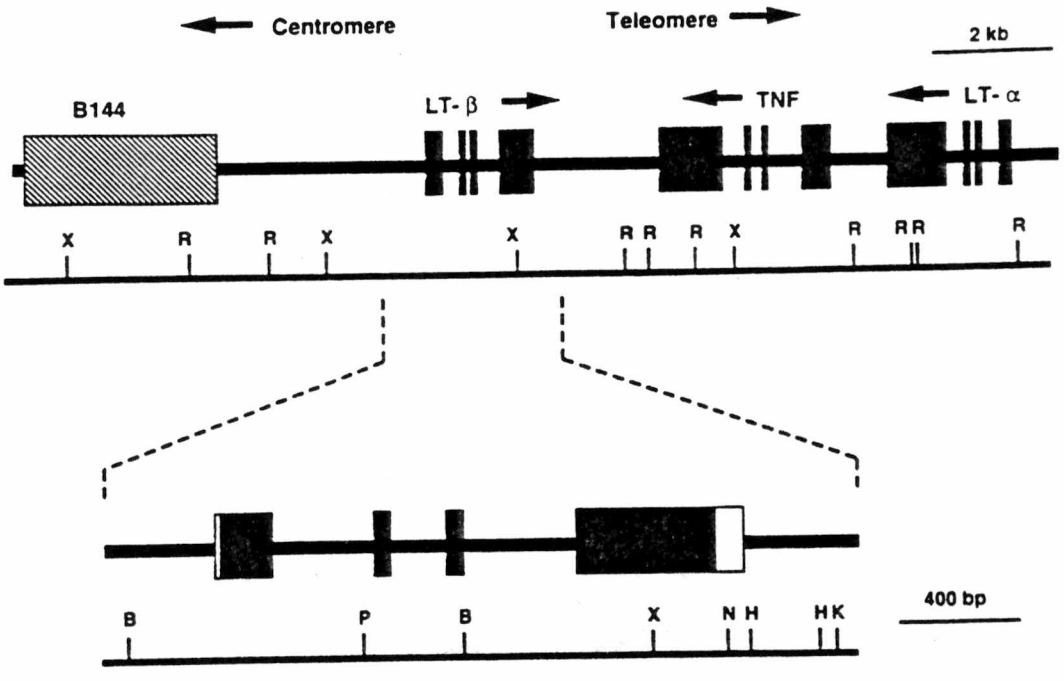


Figure 1: Schematic diagram of the gene arrangement and close proximity of the human loci for LT-α, TNF, and LT-β located on chromosome 6. The murine arrangement is quite similar although found on chromosome 17. Taken from: Browning, et. al. 1993. Lymphotoxin β, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* 72: 847-856.

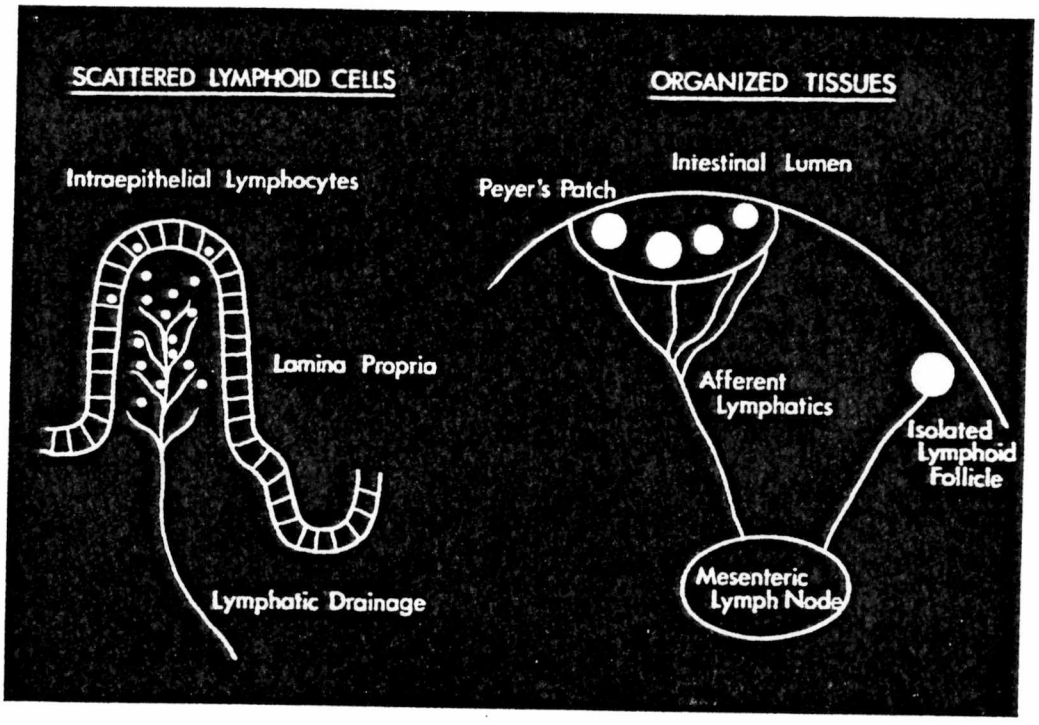
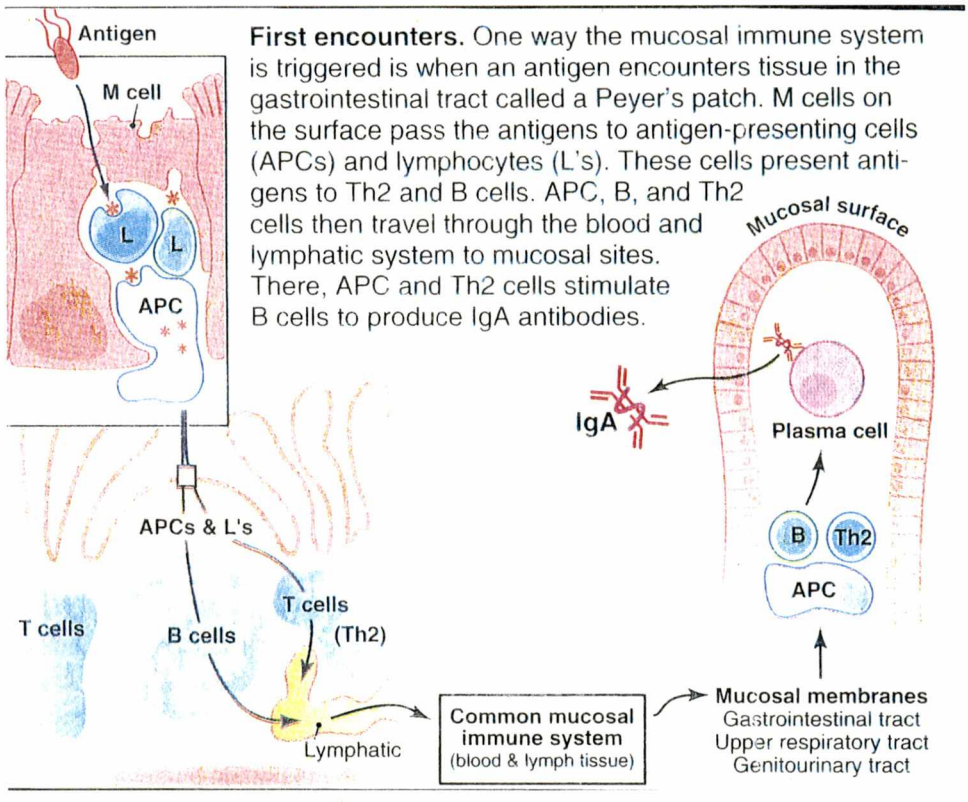


Figure 2: Schematic representation of GALT. Organized tissues include Peyer's patches and mesenteric lymph nodes while scattered lymphocytes are plentiful within the lamina propria (LPL) and among the enterocytes (intraepithelial cells, IEL). Taken from: Mowat, et. al. 1997. The anatomical basis of intestinal immunity. *Ann. Rev. Immunol.* 156: 145-166.

Figure 3: Cartoon of the currently held dogma regarding the initiation of local IgA immune responses to antigen within the intestinal tract. Typical encounters are across M cell surfaces followed by antigen delivery to lymphocytes or antigen presenting cells within the folds of the M cell. Taken from: Service, R. F., 1994. Triggering the first line of defense. *Science* 265: 1522-1524.

Figure 3



First encounters. One way the mucosal immune system is triggered is when an antigen encounters tissue in the gastrointestinal tract called a Peyer's patch. M cells on the surface pass the antigens to antigen-presenting cells (APCs) and lymphocytes (L's). These cells present antigens to Th2 and B cells. APC, B, and Th2 cells then travel through the blood and lymphatic system to mucosal sites. There, APC and Th2 cells stimulate B cells to produce IgA antibodies.

Literature Cited

1. Aggarwal, B. B., Moffat, B., and Harkins, R. N. 1984. Human lymphotoxin production by a lymphoblastoid cell line, purification, and initial characterization. *J. Biol. Chem.* 259: 686-691.

2. Aggarwal, B. B., Henzel, W. J., Moffat, B., Kohr, W. J., and Harkins, R. N. 1985. Primary structure of human lymphotoxin derived from 1788 lymphoblastoid cell line. *J. Biol. Chem.* 260: 2334-2344.

3. Ruddle, N. H., and Waksman, B. H. 1967. Cytotoxic effect of lymphocyte-antigen interaction in delayed hypersensitivity. *Science* 157: 1060-1062.

4. Gray, P. W., Aggarwal, B. B., Benton, C. V., Bringman, T. S., Henzel, W. J., Jarrett, J. A., Leung, D. W., Moffat, B., Ng, P., Svedersky, L. P., Palladino, M. A., and Nedwin, G. E. 1984. Cloning and expression of the cDNA for human lymphotoxin: A lymphokine with tumor necrosis activity. *Nature* 312: 721-724.

5. Li, C. B., Gray, P. W., Lin, P.-F., McGrath, K. M., Ruddle, F. H., and Ruddle, N. H. 1987. Cloning and expression of murine lymphotoxin cDNA. *J. Immunol.* 138: 4496-4501.

6. Gardner, S. M., Mock, B. A., Hilgers, J., Huppi, K. E., Roeder, W. D. 1987. Mouse lymphotoxin and tumor necrosis factor: Structural analysis of the cloned genes, physical linkage and chromosomal linkage. *J. Immunol.* 139: 476-483.

7. Paul, N. L. and Ruddle, N. H. 1988. Lymphotoxin. *Ann. Rev. Immunol.* 6: 407-438.

8. Nedospasov, S. A., Hirt, B., Shakov, A. N., Dobrynin, V. N., Kawashima, E., Accolla, R. S., and Jongenell, C.V. 1986. The genes for tumor necrosis factor (TNF alpha) and lymphotoxin (TNF-beta) are tandemly arranged on chromosome 17 of the mouse. *Nucleic Acids Res.* 14: 7713-7725.

9. Muller, U., Jongenell, C. V., Nedospasov, S. A., Lindahl, K., and Steinmetz, M. 1987. Tumor necrosis factor and lymphotoxin genes map close to H-2D in the mouse major histocompatibility complex. *Nature* 325: 265-267.

10. Nedospasov, S. A., Shakov, A. N., Turetskaya, R. L., Mett, V. A., Georgiev, G. P., Dobrynin, V. N., and Korobko, V. G. 1985. Molecular cloning of human genes coding for tumor necrosis factor: tandem arrangement of the alpha- and beta-genes in a short segment (16 kilobase pairs) of the human genome. *Dokl. Akad. Nauk. USSR* 286: 1487-1490.

11. Spriggs, D. R. 1991. Tumor necrosis factor: Basic principles and preclinical studies. *In:* "Biologic Therapy of Cancer", DeVita, V. T., Mellman, S., and Rosenberg, S. A., eds., Iowa State University Press, Des Moines, IA, pp. 354-392.

12. Smith, C. A., Farrah, T., and Goodwin, G. G. 1994. The TNF receptor superfamily of cellular and viral proteins: Activation, costimulation, and death. *Cell* 76: 959-962.

13. Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeman, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P. S., Kronke, M., and Mak, T. W. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell* 73: 457-467.

14. Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. 1993. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes. Nature* 364: 798-802.

15. Erickson, S. L., de Sauvage, F. J., Kikly, K., Carver-Moore, K., Pitts-Meeks, S., Gillett, N., Sheehan, K. C. F., Schreiber, R. D., Goeddel, D. V., and Moore, M. W. 1994. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* 372: 560-563.

16. Tartaglia, L. A., Pennica, D., and Goeddel, D. V. 1993. Ligand passing: The 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signalling by the 55-kDa TNF receptor. *J. Biol. Chem.* 268(25): 18542-18548.

17. Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., Hession, C., O'Brine-Greco, B., Foley, S. F., and Ware, C. F. 1993. Lymphotoxin β, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* 72: 847-856.

18. Ware, C. F., Crowe, P. D., Grayson, M. H., Androlewicz, M. J., and Browning, J. L. 1992. Expression of surface lymphotoxin and TNF on activated T, B and NK cells. *J. Immunol.* 149: 3881-3888.

19. Crowe, P. D., VanArsdale, T. L., Walters, B. N., Ware, C. F., Hession, C., Ehrenfels, B., Browning, J. L., Din, W. S., Goodwin, R. G., and Smith, C. A. 1994. A lymphotoxin-β-specific receptor. *Science* 264: 707-709.

20. De Togni, P., Goellner, J., Ruddle, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. S., Carlson, R., Shornick, L. P., Strauss-Schoenberger, J., Russell, J. H., Karr, R., and Chaplin, D. D. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264: 703-707.

21. Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D., M., Thomas, J., Kanangat, S., and Mucenski, M. L., 1995. Lymphotoxin-α-deficient mice: Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155: 1685-1693.

22. Lawton, P., Nelson, J., Tizard, R., and Browning, J. L. 1995. Characterization of the mouse lymphotoxin-β gene. *J. Immunol.* 154: 239-246.

23. Millet, I., and Ruddle, N. H. 1994. Differential regulation of lymphotoxin (LT), lymphotoxin-β (LT-β), and tumor necrosis factor (TNF-α) in murine T cell clones activated through the T cell receptor. *J. Immunol.* 152: 4336-4347.

24. Koni, P. A., Sacca, R., Lawton, P., Browning, J. L., Ruddle, N. H., and Flavell, R. A. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. *Immunity* 6: 491-500.

25. Liu, Y.-J. and Arpin, C. 1997. Germinal center development. *Immunol. Rev.* 156: 111-126.

26. Alimzhanov, M. B., Kuprash, D. V., Kosco-Vilbois, M. H., Luz, A., Turetskaya, R. L., Tarakhovsky, A., Rajewsky, K., Nedospasov, S. A., and Pfeffer, K. 1997. Abnormal development of secondary lymphoid tissues in lymphotoxin β-deficient mice. *Proc. Natl. Acad. Sci. USA.* 94: 9302-9307.

27. Gu, H., Zou, Y. R., and Rajewsky, K. 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* 73: 1155-1164.

28. Neumann, B., Luz, A., Pfeffer, K., and Holzmann, B. 1996. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J. Exp. Med.* 184: 259-264.

29. Matsumoto, M., Mariathasan, S., Nahm, M. H., Baranyay, F., Peschon, J. J., and Chaplin, D. D. 1996. Role of lymphotoxin and the type 1 TNF receptor in the formation of germinal centers. *Science* 271: 1289-1291.

30. Le Hir, M., Bluethmann, H., Kosco-Vilbois, M. H., Muller, M., di Padova, F., Moore, M., Ryffel, B., and Eugster, H.-P. 1996. Differentiation of follicular dendritic cells and full antibody responses require tumor necrosis factor receptor-1 signaling. *J. Exp. Med.* 183: 2367-2372.

31. Fu, Y.-X., Huang, G., Matsumoto, M., Molina, H., and Chaplin, D. D. 1997. Independent signals regulate development of primary and secondary follicle structure in spleen and mesenteric lymph node. *Proc. Natl. Acad. Sci.* 94: 5739-5743.

32. Pasparakis, M., Alexopoulou, L., Grell, M., Pfizenmaier, K., and Bluethmann, H. 1997. Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor. *Proc. Natl. Acad. Sci.* 94: 6319-6323.

33. Ettinger, R., Browning, J. L., Michie, S. A., van Ewijk, W., and McDevitt, H. O. 1996. Disrupted splenic architecture, but normal lymph node development in mice

expressing a soluble lymphotoxin-β receptor-IgG1. *Proc. Natl. Acad. Sci.* 93: 13102-13107.

34. Rennet, P. D., Browning, J. L., Mebius, R., Mackay, F., and Hochman, P. S. 1996. Surface lymphotoxin α/β complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184: 1999-2006.

35. Mackay, F., Majeau, G. R., Lawton, P., Hochman, P. S., and Browning, J. L. 1997. Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice. *Eur. J. Immunol.* 27: 2033-2042.

36. Neutra, M. R., Pringault, E., and Kraehenbuhl, J. P. 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* 14: 275-300.

37. LeFrancois, L. 1994. Basic aspects of intraepithelial lymphocyte immunobiology. *In:* Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGhee, J. R., and Bienenstock, J., eds. Handbook of Mucosal Immunology. San Diego: Academic Press Inc., pp: 287-298.

38. Mowat, A. M. and Viney, J. L. 1997. The anatomical basis of intestinal immunity. *Immunol. Rev.* 156: 145-166.

39. Bland, P. W. and Warren, L. G. 1986. Antigen presentation by epithelial cells of rat small intestine. II. Selective induction of supressor T cells. *Immunology* 58: 9-14.

40. Harper, H., Cochrane, L., Williams, N. A. 1996. The role of small intestinal antigen-presenting cells in the induction of T-cell reactivity to soluble protein antigens:

association between aberrant presentation in the lamina propria and oral tolerance. *Immunology* 89: 449-456.

41. Galliaerde, V., Desvignes, C., Peyron, E., Kaiserlian, D. 1995: Oral tolerance to haptens: intestinal epithelial cells from 2, 4-dinitrochlorobenzene-fed mice inhibit hapten-specific T cell activation *in vitro*. *Eur. J. Immunol.* 25: 1385-1390.
42. Guehler, S. R., Bluestone, J. A., and Barrett, T. A. 1996. Immune deviation of 2C transgenic intraepithelial lymphocytes in antigen-bearing hosts. *J. Exp. Med.* 184: 493-503.
43. Barrett, T. A., Tatsumi, Y., and Bluestone, J. A. 1993. Tolerance of T cell receptor γ/δ cells in the intestine. *J. Exp. Med.* 177: 1755-1762.
44. Mosley, R. L., Whetsell, M., and Klein, J. R. 1991. Proliferative properties of murine intestinal intraepithelial lymphocytes (IEL): IEL expressing TcRαβ or TcRγδ are largely unresponsive to proliferative signals mediated via conventional stimulation of the CD3-TcR complex. *Int. Immunol.* 3: 563-569.
45. Viney, J. L. and MacDonald, T. T. 1992. Lymphokine secretion and proliferation of intraepithelial lymphocytes from murine small intestine. *Immunology* 77: 19-24.
46. Sydora, B. C., et. al. 1993. Intestinal intraepithelial lymphocytes are activated are activated and cytolytic but do not proliferate as well as other T cells in response to mitogenic stimuli. *J. Immunol.* 150: 2179-2191.
47. Gebert, A., Rothkotter, H. J., and Pabst, R. 1996. M cells in Peyer's patches of the intestine. *Int. Rev. Cytol.* 167: 91-159.

48. Robertson, S. M. and Cebra, J. J. 1976. A model for local immunity. *Ric. Clin. Lab.* 6(3): 105-119.

49. Cebra, J. J., Kamat, R., Gearhart, P., Robertson, S. M., and Tseng, J. 1977. The secretory IgA system of the gut. *Ciba Found. Symp.* 46: 5-22.

50. Husband, A. J. and Gowans, J. L. 1978. The origin and antigen-dependent distribution of IgA-containing cells in the intestine. *J. Exp. Med.* 148: 1146-1160.

51. Jones, B., Pascopella, L., and Falkow, S. 1995. Entry of microbes into the host: using M cells to break the mucosal barrier. *Curr. Opin. Immunol.* 7: 474-478.

52. Kato T. and Owen, R. L. 1994. Structure and function of intestinal mucosal epithelium. *In:* Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGhee, J. R., and Bienenstock, J., eds. Handbook of Mucosal Immunology. San Diego: Academic Press Inc., pp: 11-26.

53. Maury, J., Nicoletti, C., Guzzo-Chanbraud, L., and Maroux, S. 1995. The filamentous brush border glycocalyx, a mucin-like marker of enterocyte hyper-polarization. *Eur. J. Biochem.* 228: 323-331.

54. Hultgren, S. J., Abraham, S., Caparon, M., Falk, P., St. Geme, J. W., and Normark, S. 1993. Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. *Cell* 73: 887-901.

55. Phillips-Quagliata, J. M. and Lamm, M. E. 1994. Lymphocyte homing to mucosal effector sites. *In:* Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGhee, J. R., and Bienenstock, J., eds. Handbook of Mucosal Immunology. San Diego: Academic Press Inc., pp: 225-239.

56. Pavli, P., Woodhams, C. E., Doe, W. F., and Hume, D. A. 1990. Isolation and characterization of antigen-presenting dendritic cells from the mouse intestinal lamina propria. *Immunology* 70: 40-47.

57. Liu, L. and MacPherson, G. G. 1993. Antigen acquisition by dendritic cells: intestinal dendritic cells acquire antigen administered orally and can prime naïve T cells *in vivo*. *J. Exp. Med.* 177(5): 1299-1307.

58. Abreu-Martin, M. T. and Torgon, S. R. 1996. Regulation of immune responses in the intestinal mucosa. *Crit. Rev. Immunol.* 16: 277-309.

59. Bargatze, R.F., Jutila, M.A., and Butcher, E.C. 1995. Distinct roles of L-selectin and integrins $\alpha 4\beta 7$ and LFA-1 in lymphocyte homing to Peyer's patch- HEV in situ: The multistep model confirmed and redefined. *Immunity* 3: 99-108 .

Part 2: Identification of immune effector sites in lymphotoxin-alpha deficient mice.

Chapter 1: Abstract

The complete lack of secondary lymphoid tissues in the lymphotoxin-alpha (LT-α) deficient (mutant) mouse allows it to be used as a model for dissecting the events involved in the development of immune responses following oral administration of immunogens. The mutant mouse lacks known gut immune inductive sites (Peyer's patches and mesenteric lymph nodes), but appears to possess the normal gut immune effector sites (the intraepithelial and lamina propria lymphocytes (IEL and LPL, respectively)). Additionally, the mutant mouse possesses a spleen which may provide some effector and perhaps inductive help following oral administration of certain antigens. Specifically, the mutant mouse develops a marked splenomegaly following oral administration of *Salmonella typhimurium* strain BRD847 and generates both serum and fecal antigen specific IgG and IgA responses. However, splenectomized mutant mice are still capable of mounting these specific immunoglobulin responses (Part 5). Therefor, we conclude and show in these studies that mutant mice possess not only alternate mucosal immune induction sites but very likely utilize the LPL compartment in conjunction with splenic cell populations as major effector sites.

Chapter 2: Introduction

The intestinal mucosa is in constant contact with foreign proteins and microorganisms. Although many of these antigens are harmless, large quantities gain entry to the body necessitating some type of host response. The most important protective host response at mucosal surfaces is the local production of secretory IgA. Antigen uptake occurs both through M cells overlying Peyer's patches (PP) or across normal epithelium overlying the lamina propria (LP). The induction of IgA responses to antigens encountered across the gut mucosa is generally thought to occur in specialized components of the gut-associated lymphoid tissue (GALT), the PP and mesenteric lymph nodes (MLN). Within the PP are located naïve B and T lymphocytes which, following appropriate antigenic stimulation, travel within lymphatic vessels to the MLN where they receive appropriate maturational and costimulatory signals necessary for clonal expansion. From the MLN, these newly activated cells course through the lymphatics to the vascular compartment which allows specific homing back to the LP compartment where they may carry out their specific effector functions. The LP is a major mucosal effector site possessing all cellular components necessary for both humoral and cell-mediated immune responses (1). Within the LP, lymphoid cells are selectively retained and interact such that B cells undergo clonal expansion under the influence of specific antigen stimulation and with the aid of local T cells and cytokines, to become IgA secreting plasma cells (2, 3). Lymphocytes within the intraepithelial (IEL) compartment also serve effector functions but are generally thought to be regulatory in nature with the majority of cells being of the $CD8^+$ $\alpha\beta$TCR variety (4).

The LT-α deficient (mutant) mouse has no PP or MLN and yet is capable of mounting specific mucosal IgA responses to several orally administered antigens (see subsequent chapters). While it is possible that antigen entry occurs both through M cells (their absence has yet to be confirmed in mutant mice) and across enterocytes, the location of inductive sites is unresolved. The IEL and LP compartments are intact in mutant mice and we suspect the LP serves to harbor both inductive and effector lymphocyte (LPL) populations in mutant mice. Interestingly, the spleens of mutant mice undergo significant enlargement both with age and following oral administration of certain bacterial strains. Therefor, the spleen may additionally serve as an alternate effector and perhaps inductive site for antigen specific responses in mutant mice.

To explore the location of possible effector sites in mutant mice, a severe combined immunodeficient (*scid)* mouse reconstitution model was employed in which whole spleen, IEL, or LPL cell populations from mutant or wild-type mice were used as donor cell populations. Our data indicate that cells from both the spleen and LP compartments of mutant mice provide all components necessary for the generation of antigen specific systemic and mucosal humoral immune responses. Mutant mice therefor utilize alternate components of the GALT, and possibly the spleen, for the induction and development of mucosal immune responses.

Chapter 3: Materials and Methods

Mice

All mice used in this and all described experiments descended from founder mutant mice developed by targeted disruption of the LT-α gene as described elsewhere (5). F_1 homozygous mutant female mice were bred to C57BL/6 wild-type male mice obtained from Jackson Laboratories (Bar Harbor, ME). Resulting heterozygous female offspring were bred back to their fathers for to achieve congenicity. All mice used in the experiments described in this section were the progeny of mutant or wild-type offspring from the F3 or F4 congenic generation. The phenotypes of all mice were confirmed by a 40 cycle polymerase chain reaction on prepared genomic DNA from tail tissue using two primer sets specific to exons 1 and 3 of the LT-α gene and primers specific to the extreme 5' and 3' ends of the neomycin insert. Preparation of tail DNA involves an overnight Proteinase K digestion followed by extraction with phenol and ethanol (personal communication, R. P. Woychick, PhD, Case Western University, Cleveland, Ohio). The primer sequences follow:

LT-α sense: 5'-GCC CAG GAC CCC GCA CAG C-3'

LT-α antisense: 5'-GGA GAA GCG GAC ACC AGA GA-3'

Neomycin sense: 5'-TCA GCG CAG GGG CGC CCG GTT CTT T-3'

Neomycin antisense: 5'-ATC GAC AAG ACC GGC TTC CAT CCG A-3'

The PCRs were performed on an MJ Research PTC-100 thermocycler (Watertown, Mass.) using the following program repeated 39 times:

Denature: 94 °C for 30 seconds

Anneal: 60 °C for 30 seconds

Extension: 72 °C for 1 minute 30 seconds

The PCR products were loaded onto 1.5% agarose gels and electrophoresed to generate adequate band separation. Using these primers, wild-type (w.t.) mice possess only the 720 base pair (bp) band indicating an intact LT-α gene while mutant mice possess only the 298 bp band specific for the neomycin insert. Heterozygous mice possess both bands. **Figure 1** (pages 44- 45) illustrates typical PCR results.

All mice were additionally confirmed to be LT-α deficient upon termination by gross inspection of viscera, enlarged spleens relative to w.t. controls, and the lack of all secondary lymphoid tissues. **Figures 2a and b** (pages 46-48) illustrate typical necropsy findings of mutant and w.t. mice.

C57BL/6 *scid* founder breeding mice were a kind gift from Dr. J. Russell Lindsey (University of Alabama, Birmingham).

All mice were housed in microisolator cages and maintained in a barrier facility at the Walters Life Sciences Building, University of Tennessee, Knoxville, and handled in accordance with institutional guidelines.

Isolation of donor cell populations

Mutant and w.t. mice between the ages of 5 and 8 weeks were terminated for the collection of the following tissues: whole splenocytes, IEL, and LPL. Spleens were aseptically removed, minced with scissors, and gently pressed through a wire mesh screen into cold RPMI 1640 medium (Gibco/BRL, Gaithersburg, MD) supplemented

with 10% heat inactivated fetal bovine serum (Gibco/BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Red blood cells were lysed by incubating cells for 15 minutes in a 37 °C water bath in ammonium chloride-Tris solution and remaining white blood cells were washed and suspended to 1.0×10^7 cells/ml.

IEL and LPL were isolated following sequential incubation and digestion steps. Specifically, small intestinal segments were aseptically removed and placed in cold Ca^{2+} and Mg^{2+} free sterile 1x physiologic buffered saline (PBS) (solution 1). Segments from w.t. mice were carefully inspected for PPs which were removed. Segments were cut longitudinally along the mesenteric border, freed of all vascular and mesenteric attachments, washed thoroughly in solution 1, and cut into 5 mm pieces. These pieces were then placed in complete RPMI media containing 5 mM EDTA and agitated at 37 °C for 30 minutes. Following incubation, supernatants, containing epithelial cells and IELs, were collected. This process was repeated a total of 4 times. The pooled supernatants were centrifuged at 1000 x *g* for 10 minutes, cell pellets collected and resuspended in complete RPMI media, counted using a hemocytometer, and maintained at 4 °C until further processing. The remaining intestinal segments were placed in complete RPMI media containing 1.5 mg/ml Dispase (Gibco/BRL, Gaithersburg, MD) and incubated with agitation for 60 minutes at 37 °C. Following incubation, supernatants were collected and saved and the media replaced over intestinal segments. The incubation and supernatant collection steps were repeated a total of 4 times. Finally, supernatants were collected and centrifuged as above. Cell pellets, which consisted of LP cell populations devoid of supporting structures, were resuspended in complete media and counted. All cell

populations were subjected to Percoll (Sigma, St. Louis, MO) gradient centrifugation and the banded cells between to 40-100% interface were collected, counted, and adjusted to 1.0×10^7 cells/ml. IEL and LPL populations from 8 naïve mutant and 9 naïve w.t. mice were pooled to allow significant cell numbers for reconstitution of *scid* recipients. **Table 1** (page 55) summarizes the isolated cell populations from donor mice and **Table 2** (page 55) indicates the concentrations of cells used to reconstitute *scid* mice.

Preparation of recipient mice

Recipient *scid* mice between the ages of 6 and 8 weeks were lethally irradiated (whole body dose of 750 rads) 6 hours prior to receiving reconstituting cell populations. Mice were reconstituted intraperitoneally with the cell populations indicated in **Table 2** at the listed cell concentrations in 500 µl sterile 1X HBSS at room temperature (RT). The IP route of administration was chosen based on preliminary work comparing the efficacy of intravenous versus IP delivery of different concentrations of whole splenocytes.

Identification of donor cell subsets

An aliquot of each donor cell population from both mutant and wild-type mice were prepared for fluorescence activated cell scanning (FACS) analysis. Cells were adjusted to 1.0×10^6 cells/ml in FACS staining buffer (0.1% sodium azide and 5% fetal calf serum in PBS) containing appropriately labeled phycoerythrin (PE) and fluoresceinisothiocyanate (FITC) anti-mouse antibodies (PharMingen) at a concentration

of 5 ng/μl. The following cell populations were identified: CD_4 and CD_8 T cells, γδ and αβ T cell receptor (TCR) bearing T cells, and B and T cells (B220 versus Thy1.2).

Inoculation of recipient mice

Salmonella typhimurium strain BRD847 has been well characterized (6,7) and was used under agreement with Medeva PLC (Surrey, UK) to orally immunize mice in this and subsequent studies. BRD847 is an *aroA* mutant derived from the parental strain SL1344 and modified to express the fragment C of tetanus toxin (ToxC) under anaerobic conditions, driven by the *nirB* promoter. Cultures were added to Luria-Bertani (LB) broth with 50 μg/ml ampicillin and incubated 16 hours at 37 °C. The culture was then diluted 1:50 in fresh LB broth with ampicillin and cultured for an additional 4 hours at 37 °C. The bacteria were then pelleted at 6000 x *g* at 4 °C and resuspended in 5 ml ice cold 1x PBS. Mice were held off feed and water for 3 hours, placed under general inhalant anesthesia (Metofane, Mallinckrodt Veterinary, Inc., Mundelein, IL), and dosed orally with 100 μl (approximately 1.0×10^9 colony forming units (CFU)) of the bacterial culture via an orogastric feeding needle. Bacterial cultures were subsequently serially diluted and plated on LB-ampicillin agar to confirm CFU given to mice. Both naïve and reconstituted *scid* mice were inoculated.

Collection of samples from inoculated mice

Serum and fecal samples were collected at the time of reconstitution and at 1, 3, and 4 weeks post-reconstitution. Blood samples were obtained via capillary tube

puncture of the retro-orbital venous sinus of anesthetized mice. Serum was separated following sample centrifugation and stored at -20° C until analysis. Individual fecal samples were collected from mice, weighed, diluted in 1 ml 0.1% sodium azide in 1x PBS per 100 mg of feces, and vortexed for 15 minutes. Fecal slurries were then centrifuged for 8 minutes at 3000 x *g*, supernatants collected and stored at –20 °C until enzyme-linked immunosorbent assays (ELISA) could be performed.

Determination of immunoglobulin responses in inoculated mice

Total and ToxC specific serum IgG and fecal IgA responses were evaluated by ELISA. **Table 3** (page 56) lists reagents used. Coating antibodies were diluted in carbonate buffer, pH 9.75, and incubated 14-18 hours at 4 °C. Non-specific protein binding was blocked using 3% nonfat dry milk incubated for 2 hours at 37 °C. Primary, secondary, and tertiary antibodies were incubated at 37 °C for 2 hours, 1 hour, and 45 minutes, respectively. Plates were washed three times in 1x PBS containing 0.2% Tween-20 (Sigma, St. Louis, MO). Serum from w.t. mice and *scid* mice reconstituted with w.t. cell populations was diluted 1:5000 for total IgG and 1:250 for ToxC specific IgG. Serum from mutant mice and *scid* mice reconstituted with mutant cell populations was diluted 1:2500 for total IgG and 1:100 for ToxC specific IgG. Fecal samples from w.t. mice and *scid* mice reconstituted with w.t. cell populations were diluted 1:400 for total IgA and 1:50 for ToxC specific IgA. Fecal samples from mutant mice and *scid* mice reconstituted with mutant cell populations were diluted 1:200 for total IgA and 1:20 for ToxC specific IgA. All samples from unreconstituted *scid* mice were used neat.

Chapter 4: Results

Comparison of donor cell populations

As shown in **Figure 3** (page 49-50), the percentage of cells from the various compartments do not differ significantly between mutant and w.t. mice. CD_4^+ cells with αβ TCRs and $B220^+$ B cells dominate the total splenocyte populations of both mutant and w.t. mice. The IEL compartment of both mutant and w.t. mice is dominated by CD_8^+ TCR T cells. The LPL compartment of mutant mice shows an increased percentage of $B220^+$ B cells but fairly equivalent numbers of the different T cell subsets, all expressing αβ TCRs.

Comparison of immune responses in recipient mice

Total and ToxC specific IgG and IgA responses as determined by ELISA 3 weeks post-reconstitution are presented in **Figures 4 and 5** (pages 51-54), respectively. As seen in Figure 4a and evidenced by low total serum IgG levels, there was poor reconstitution of *scid* mice with both mutant and w.t. cells. This same scenario holds true for mucosal responses as determined by fecal IgA levels when comparing reconstituted *scid* mice to intact mutant or w.t. mice. However, as shown in Figures 5a and b, those cells which did reconstitute and persist did respond to orally administered BRD847 with ToxC specific immunoglobulin production of both the IgG and IgA classes. ToxC specific serum IgG and fecal IgA was undetectable in all naïve mice and *scid* mice reconstituted with IEL populations. Reconstitution with LPL populations resulted in low but measurable total and ToxC specific serum IgG and fecal IgA production. Interestingly, there is little

difference in the ToxC specific IgG responses of *scid* mice reconstituted with either mutant or w.t. LPL or whole splenocytes. This may indicate a lack of impairment of homing ability and function of cells derived from these compartments in mutant mice. Reconstitution with whole splenocytes from either mutant or w.t. donors provided superior restoration of mucosal IgA production than did reconstitution with IEL or LPL cell populations (Figure 4b and 5b). Total IgA production in *scid* mice reconstituted with either mutant or w.t. LPL cells was less than that observed in naïve mutant mice. Interestingly, total fecal IgA levels in *scid* mice reconstituted with mutant cell was greater than levels from naïve mutant mice. This provides further evidence that cell homing and function are unimpaired but cellular colocalization necessary for costimulation may be impaired in mutant mice.

While the levels of ToxC specific fecal IgA was quite low in mice reconstituted with LPL from both mutant and w.t. donors, the fact that any antibody was measured suggests that the LPL compartment possesses the appropriate components necessary for the generation of antigen specific mucosal immune responses.

Chapter 5: Discussion

This study was undertaken as part of an attempt to identify alternate mucosal induction and effector sights. A reconstitution model provided the best means of separating donor cell populations and the *scid* recipient, because it lacks endogenous mature B and T cell populations, provided a means of determining immune responses specifically resulting from donor cell population activation. The *scid* mouse possesses endogenous APCs, including normal gut mucosal epithelium, thereby providing an induction site source to the transferred lymphocyte populations (8). There are no published reports on the mucosal immune responsiveness in LT-α deficient mice. We have observed that these mice are capable of mounting specific immune responses to a variety of orally administered immunogens in spite of lacking PP and MLN, normal immune induction sights. Suspecting the spleen as a major inductive or effector sight, we were surprised to see very little difference in mucosal immune responses in intact and splenectomized mutant mice (see Part 5). Further, in two separate reconstitution models (Part 4), we found that mucosal immune responsiveness was determined by the recipient genotype and this responsiveness could not be augmented by reconstituting mutant mice with w.t. cells. Normal inductive sites could not be recreated *de novo* however existing inductive sites were apparently functional but less effective. The data provided in this study allows some speculation of immune compartment components in the mutant mouse. Specifically, the LPL compartment appears to provide all cellular components necessary for the development of a specific immune response to an orally administered antigen. The IEL compartment could not provide these same components. Further, there

was very little difference in the specific responses generated in recipients of mutant or w.t. LPL. The mutant phenotype thus does not appears to extend to the LPL compartment and this may well be the major effector site in mutant mice for the development of specific mucosal immunoglobulin responses. Mutant mice produce reduced levels of mucosal IgA following oral antigen administration because they lack lymphoid tissue organization and therefor the intimate cell-cell contact between inductive (PP and MLN) and effector (IEL and LPL) tissues provided by the M cell-PP unit. Although enterocytes are quite capable of antigen uptake, antigens presented to professional APCs within the LP by this route may result in suppression and not generation of immune responses. The presence of IEL within the enterocyte layer and the inability of IEL to reconstitute *scid* mice is likely due to the paucity of B cells but may be due to the regulatory nature of cells within this compartment.

Finally, although the data presented here does not clearly identify where inductive events occur in the GALT of mutant mice, the idea that mutant mice compensate for the lack of organized mucosal inductive and effector tissue by cells within the spleen and LP is supported.

Figure 1: Results of typical PCR reaction using both LT-α and neomycin primers in a 40-cycle reaction on tail (genomic) DNA. Lane 1 contains the 1kB ladder used as a reference for sequence size. Lane 2 contains the internal control, β-actin template and primers; Lane 3 contains water and serves as a negative control; Lanes 4 and 5 contain tail DNA from mutant mice thus yielding only the 298 base pair neomycin insert band; and Lanes 6 and 7 contain tail DNA from heterozygous mice thus yielding the 720 base pair band typical of the wild-type LT-α allele as well as the neomycin band. Tail DNA from a homozygous wild-type mouse would yield only the 720 base pair band.

Figure 1

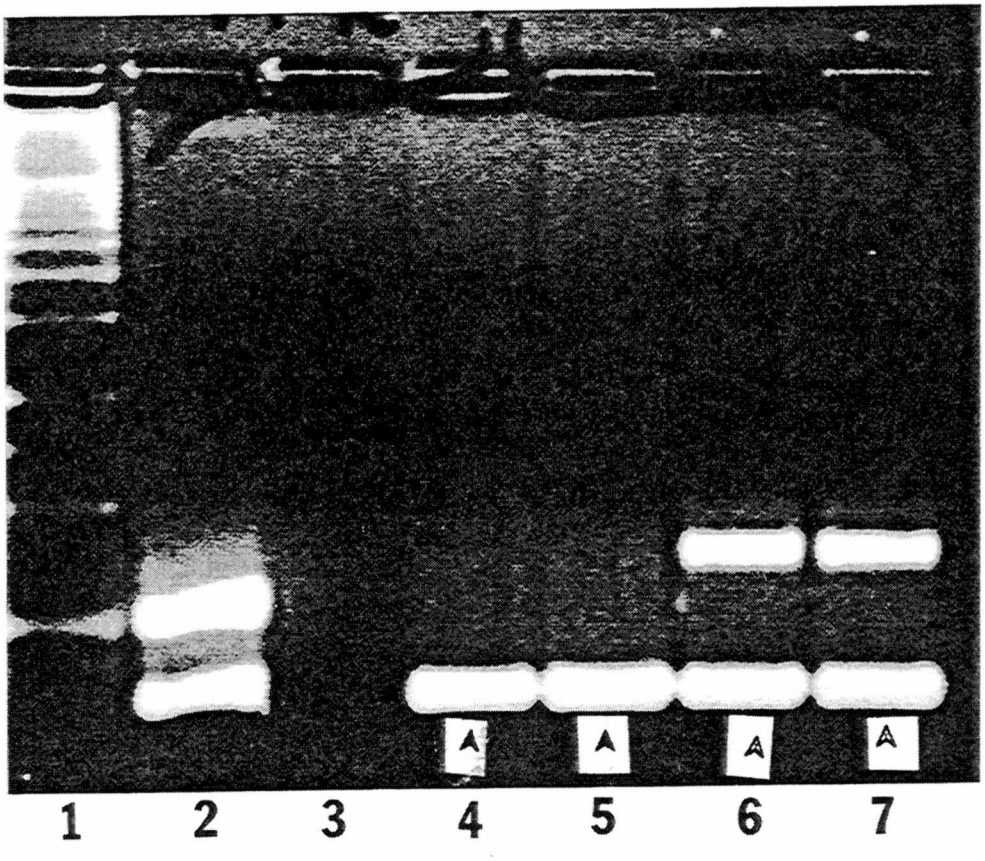


Figure 2: Gross findings of LT-α deficient mice. An inspection of the abdominal cavities of a wild-type (a) and an LT-α deficient mouse (b) reveals the lack of secondary lymphoid tissues and enlarged spleen which are common to the mutant phenotype. L = liver, S = spleen, M = mesenteric lymph nodes, and P = Peyer's patches.

Figure 2a

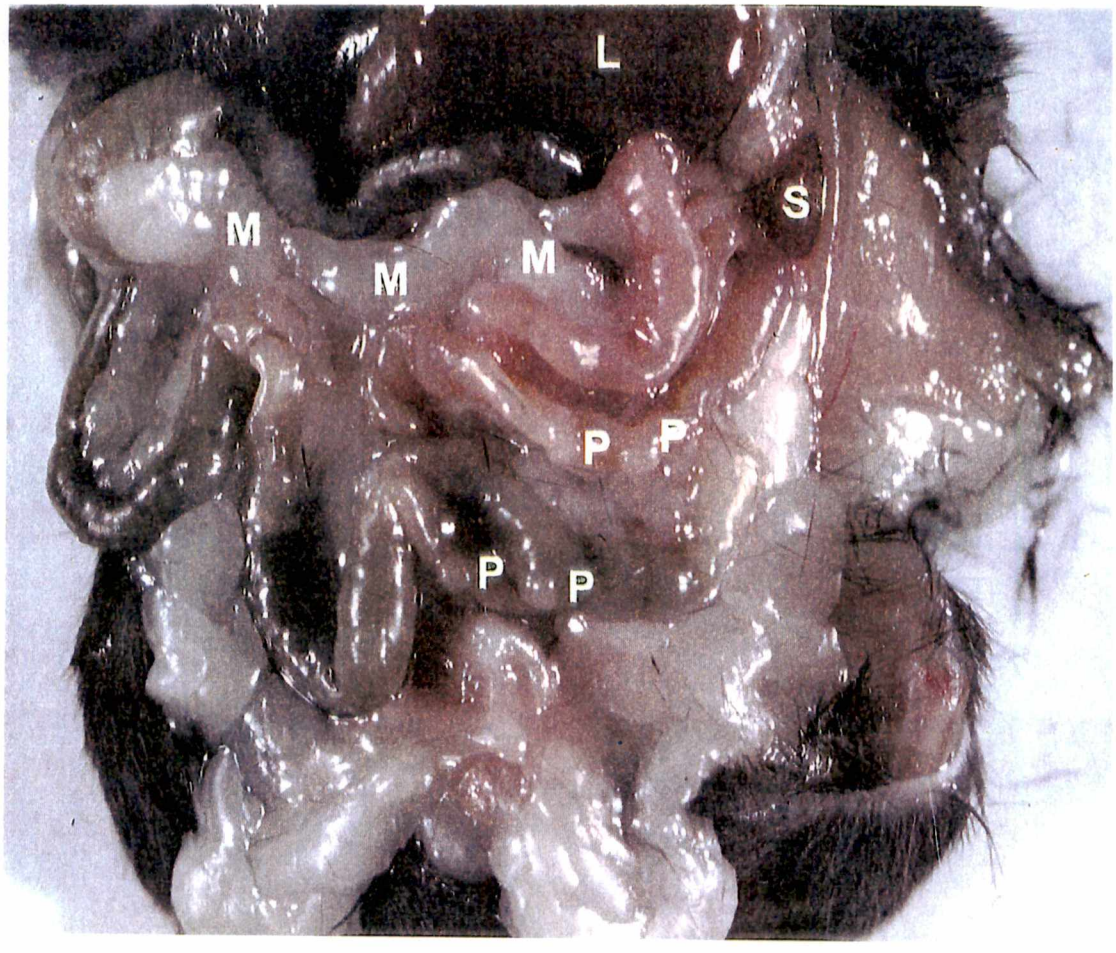


Figure 2b

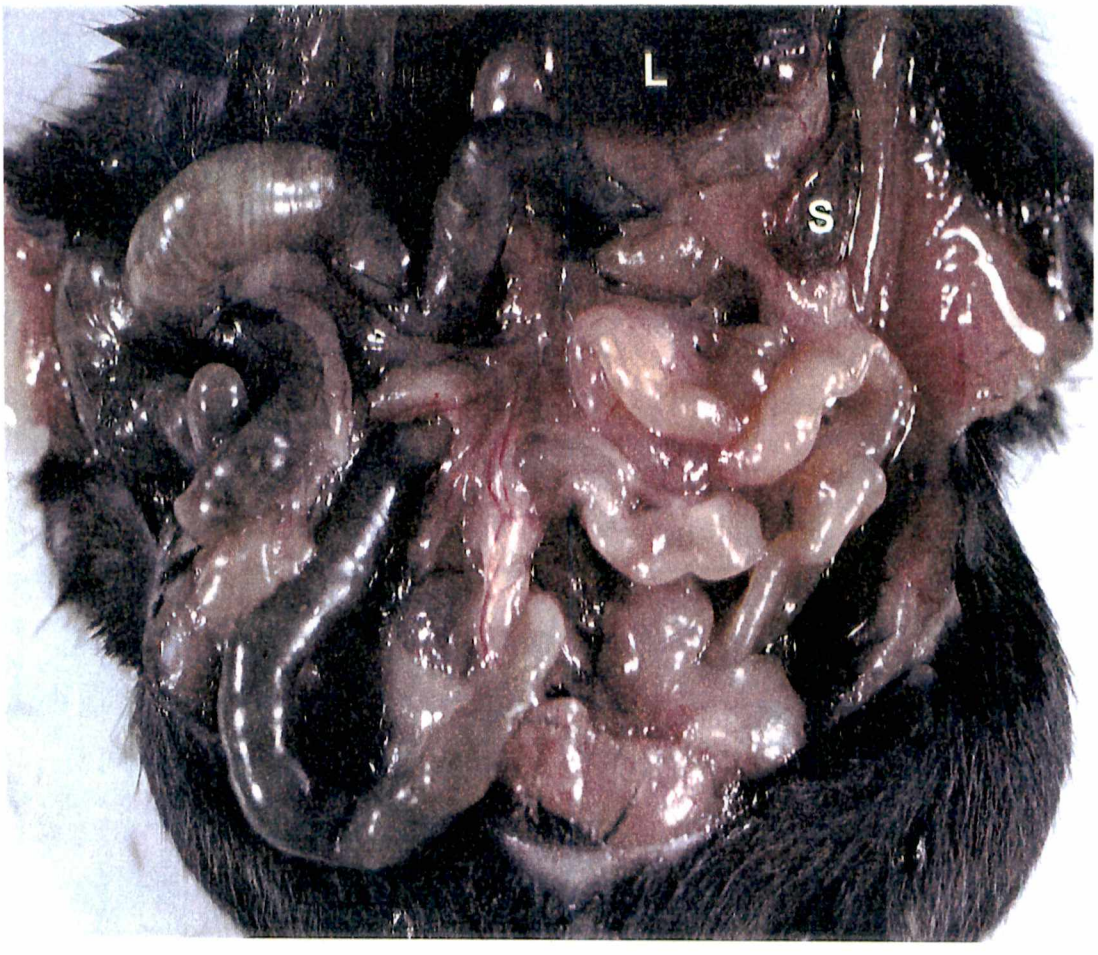


Figure 3: Comparison of donor cell populations from various compartments as determined by FACS analysis. Values are expressed as percentages of total cells analyzed. -/- = mutant, +/+ = wild-type; IEL = intraepithelial compartment; LPL = lamina propria lymphocyte compartment. The different T cell subsets and T cells expressing different receptors were assessed and compared to B220 expressing cells.

For -/- (mutant) spleen, IEL and LPL, n = 8

For +/+ (wild-type) spleen, IEL and LPL, n = 9

No significant differences were detected between any -/- or +/+ compartments.

Figure 3

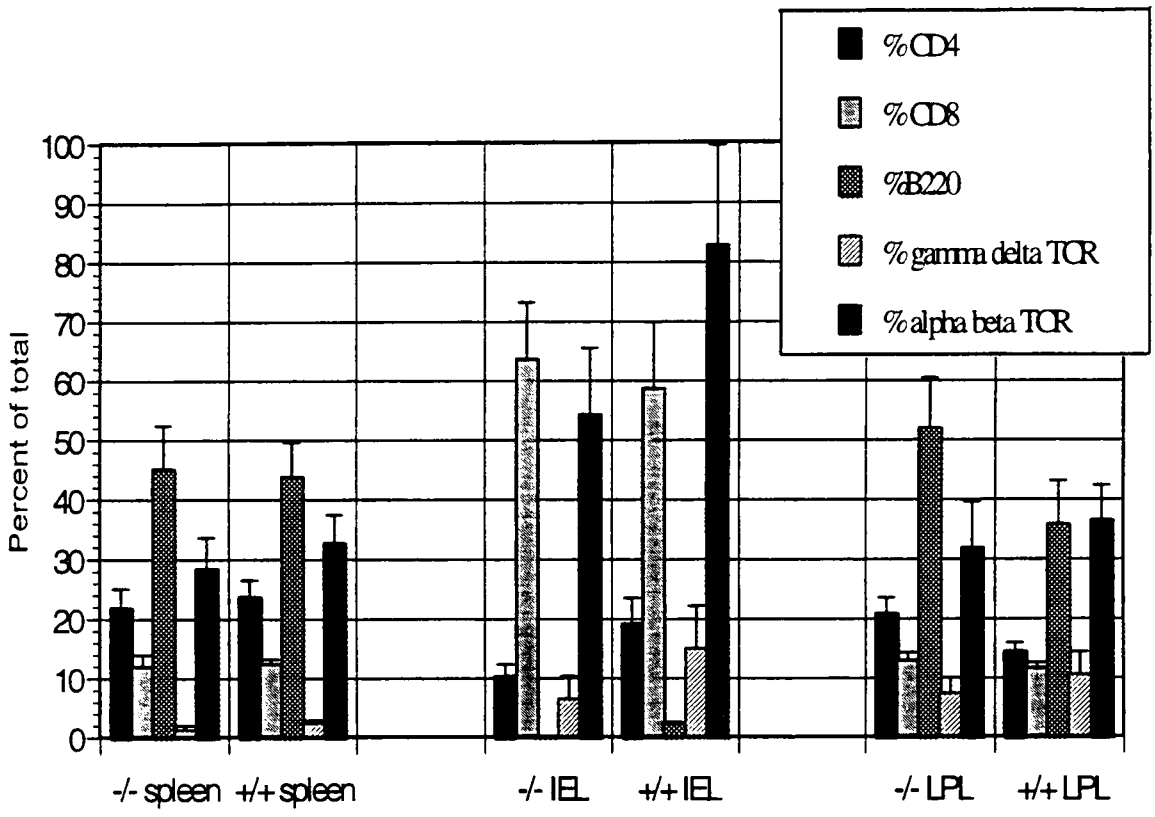

%CD4
%CD8
%B220
%gamma delta TCR
%alpha beta TCR
Percent of total
100
90
80
70
60
50
40
30
20
10
0
-/- spleen
+/+ spleen
-/- IEL
+/+ IEL
-/- LPL
+/+ LPL

Figure 4: Mean total serum IgG (a) and fecal IgA (b) ELISA results of variously reconstituted *scid* mice. "*Scid*/....." indicates "recipient/the donor population". -/- = mutant mouse, +/+ = wild-type mouse, IEL = intraepithelial compartment; LPL = lamina propria lymphocyte compartment, immunized = orally dosed with *S. typhimurium* strain BRD847 at Day 0, Day 7, and Day 21 of reconstitution. Significant differences ($p < 0.005$) were seen in the antibody responses of *scid* mice reconstituted with either IEL or LPL populations when compared to those responses generated by *scid* mice reconstituted with whole splenocytes.

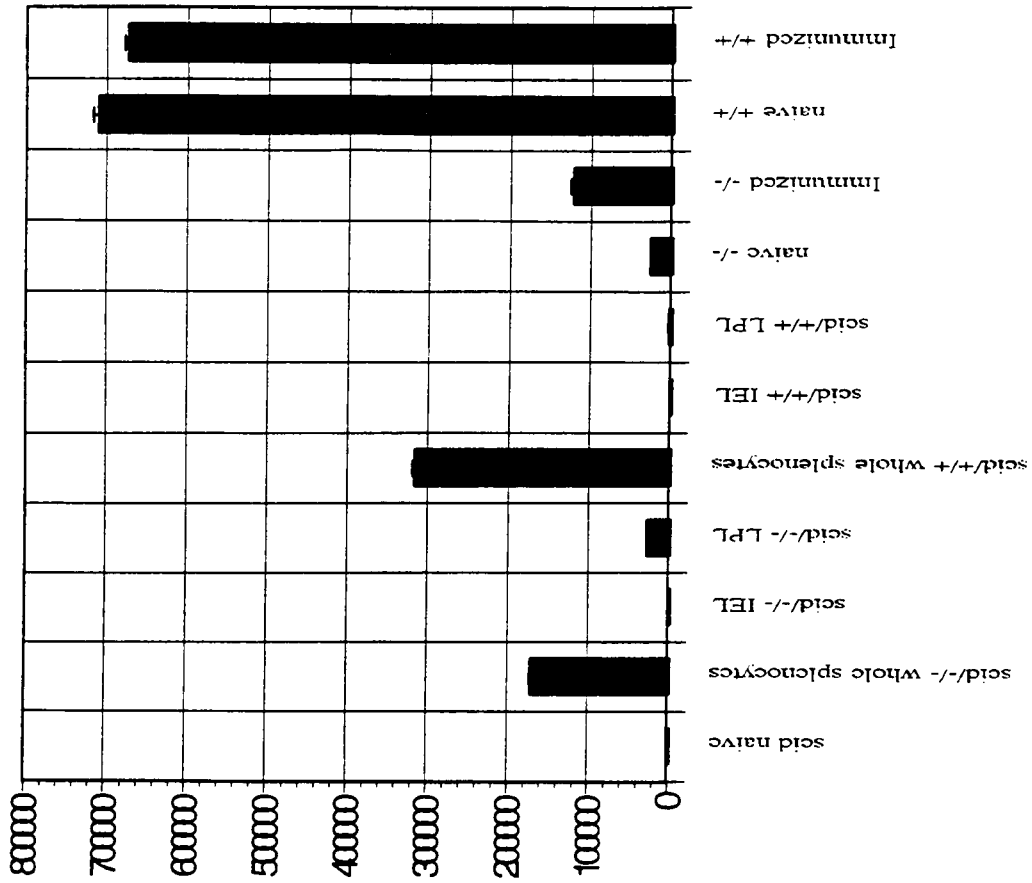


Figure 4b Mean total fecal IgA, ng/ml

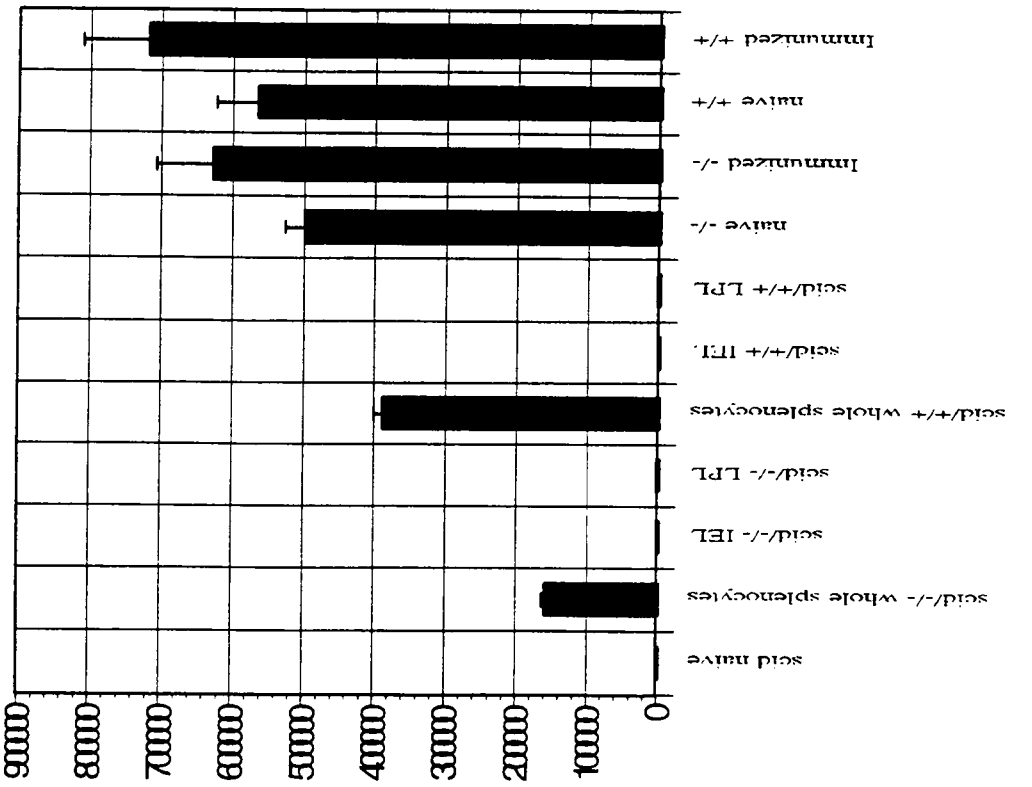


Figure 4a Total serum IgG, ng/ml

Figure 5a and b: Detection of ToxC specific fecal IgA (a) and serum IgG (b) responses in *scid* mice reconstituted with various donor cell populations. -/- = mutant mouse, +/+ = wild-type mouse, IEL = intraepithelial compartment; LPL = lamina propria lymphocyte compartment, immunized = orally dosed with *S. typhimurium* strain BRD847 at Day 0, Day 7, and Day 21 of reconstitution. The LPL compartment contains sufficient cell types to functionally repopulate the empty lymphoid compartments of *scid* mice.

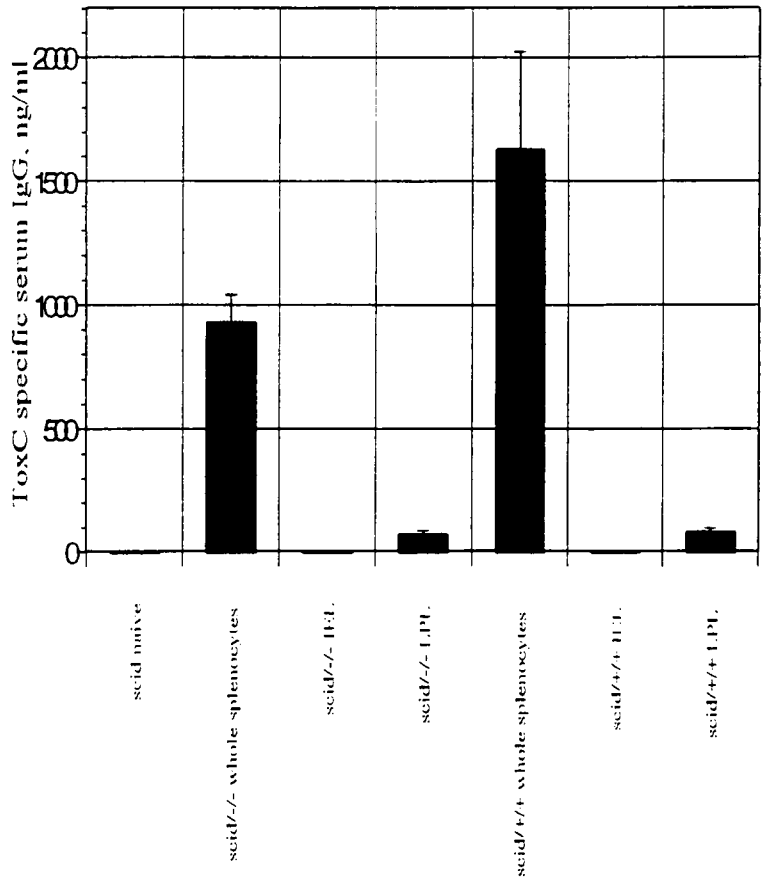


Figure 5b

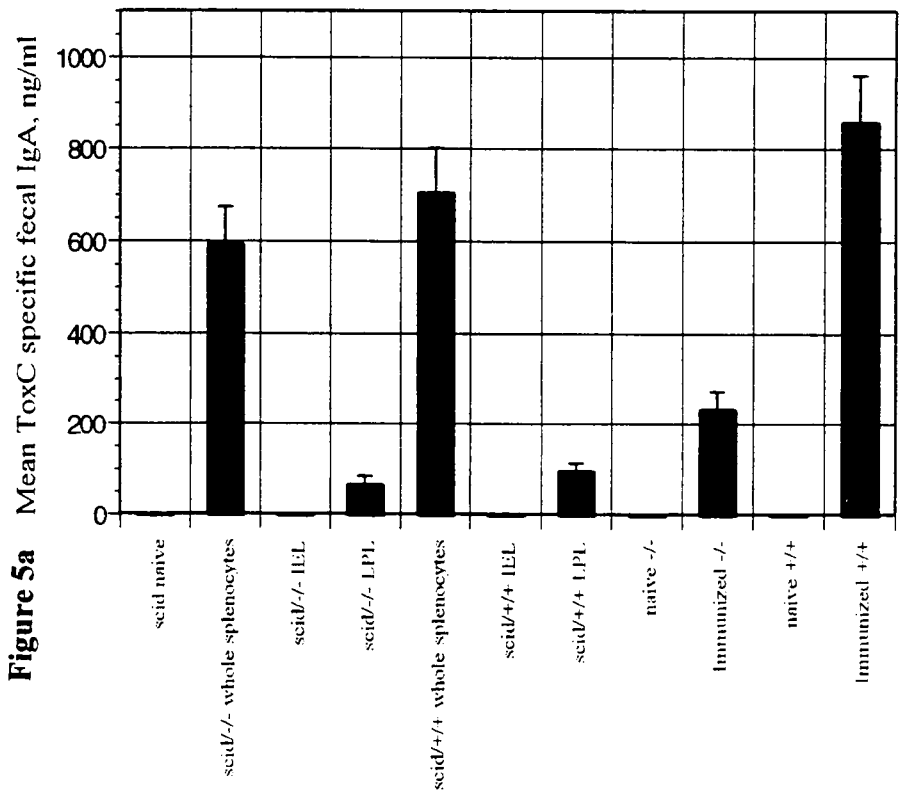


Figure 5a

Table 1: Average cell counts per mouse of isolated donor cell populations. Mutant mice harbor increased cell numbers in all compartments

Mouse Genotype	Whole splenocytes	IEL	LPL
Mutant	**4.1×10^7**	**5.9×10^6**	**3.3×10^6**
Wild-type	**3.5×10^7**	**1.2×10^6**	**1.6×10^6**

Table 2: Concentration of donor cells injected to *scid* recipients from the indicated compartments.

Donor Genotype	Whole splenocytes	IEL	LPL
Mutant	**1.0×10^7**	**3.5×10^6**	**4.0×10^6**
Wild-type	**1.0×10^7**	**3.5×10^6**	**4.0×10^6**

Table 3: ELISA reagents used for immunoglobulin concentration determination.

Coating Antibody And Dilution	Standard Antibody And Dilution	Secondary Antibody And Dilution	Tertiary Antibody And Dilution
Goat anti-mouse IgM, Jackson, 115-005-075, 1.7 mg/ml. Diluted 1:1000	Mouse myeloma IgM, MOPC-104E, Sigma, M-5170, 1.0 mg/ml. Diluted 1:4000	HRP-rabbit anti-mouse IgM, Zymed, 61-6820 , Diluted 1:2000	None
Goat anti-mouse IgG, So. Biotech. Assoc., 1030-01, 1.0 mg/ml. Diluted 1:1000	Mouse IgG- UNLB, So. Biotech. Assoc., 107-01, 0.5 mg/ml. Dilute 1:5000	HRP-goat anti-mouse IgG, So. Biotech. Assoc., 1030-05, Dilute 1:2000	None
Goat anti-mouse IgG_1, So. Biotech. Assoc., 102-01, 1.0 mg/ml. Diluted 1:1000	Mouse IgG_1- UNLB, So. Biotech. Assoc., 102-01, 1.0 mg/ml. Diluted 1.25:5000	HRP-goat anti mouse IgG_1, So. Biotech. Assoc., 1070-05 Diluted 1:2000	None
Goat anti-mouse IgG_{2a}, So. Biotech. Assoc., 103-01, 1.0 mg/ml. Diluted 1:1000	Mouse IgG_{2a}- UNLB, So. Biotech. Assoc., 1030-01, 1.0 mg/ml Diluted 1.25:5000	HRP-goat anti-mouse IgG_{2a}, So. Biotech. Assoc., 1080-05 Dilute 1:2000	None
Rabbit anti-mouse IgA, Zymed, 61-6700, 0.5 mg/ml. Diluted 1:250	Purified Mouse myeloma IgA, Zymed, 02-6500 1 mg/ml Dilute 1:10,000	Biotin-goat anti-mouse IgA, Zymed, 62-6740, Dilute 1:2000	Peroxidase-conjugated streptavidin, Jackson, 016-030-084, 1 mg/ml, Dilute 1:2000.

Literature Cited

1. McGhee, J. R., Mestecky, J., Dertzbaugh, M. T., Eldridge, J. H., Hirasawa, M., and Kiyono, H. 1992. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* 10(2): 75-88.
2. Mestecky, J. and McGhee, J. R. 1987. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv. Immunol.* 40: 153-159.
3. McGhee, J. R., Mestecky, J., Elson, C. O., and Kiyono, H. 1989. Regulation of IgA synthesis and immune response by T cells and interleukins. *J. Clin. Immunol.* 9: 175-182.
4. Mowat, A.M^{c}I., and Viney, J.L. 1997. The anatomical basis of intestinal immunity. *Immunol. Rev.* 156: 145-166.
5. Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D., M., Thomas, J., Kanangat, S., and Mucenski, M. L., 1995. Lymphotoxin-α-deficient mice: Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155: 1685-1693.
6. Chatfield, S. N., Charles, I. G., Makoff, A. J., Oxer, M. D., Dougan, G., Pickard, D., Slater, D., and Fairweather, N. F. 1992. Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single dose oral tetanus vaccine. *Bio/Technology* 10: 888-892.
7. Karem, K. L., Chatfield, S., Kuklin, N., and Rouse, B. T. 1995. Differential induction of carrier antigen-specific immunity by *Salmonella typhimurium* live-

vaccine strains after single mucosal or intravenous immunization of BALB/c mice. *Infect. Immun.* 63(12): 4557-4563.

8. Bosma, M. J. and Carroll, A. M. 1991. The SCID mouse mutant: Definition, characterization, and potential uses. *Annu. Rev. Immunol.* 9: 323-350.

Part 3: Identification of select lymphocyte homing molecules and vascular addressins in lymphotoxin-alpha deficient mice.[1]

1. Submitted for publication to BLOOD: manuscript number 7-98-5-171.

Chapter 1: Abstract

The transmigration of lymphocytes across vascular endothelium is critical for the localization of lymphocytes to lymph nodes in both naïve and immune reactive states. Mice deficient in lymphotoxin-alpha (LT-α) lack all peripheral and gut associated lymph nodes. Several investigators report normal function and homing ability of lymphocytes from these mice yet information regarding cell adhesion molecules and counterpart vascular addressins is lacking. The phenotype of LT-α deficient mice may, therefor, include defective expression or recognition of select lymphocyte homing and vascular addressin molecules. Fluorescent activated cell sorting and immunohistochemistry of paraffin embedded tissues were used to investigate the expression of several such molecules. No difference was detected in the tissue expression of MADCAM-1, PNAd, ICAM-1, PECAM-1, or $\alpha_4\beta_7$ integrin between wild-type and LT-α deficient mice. Differences were observed, however, in the levels of L-selectin expression between aged LT-α deficient and wild-type mice. The phenotype of the adult LT-α deficient mouse is unlikely the result of impaired expression of lymphocyte homing and vascular addressin molecules studied here. Nevertheless, differences in the expression of lymphocyte homing molecules may explain the development of ectopic lymphoid tissues in aged LT-α deficient mice.

Chapter 2: Introduction

The lymphotoxin-alpha (LT-α) deficient mouse provides interesting study material for the cellular interactions required for the development of lymphoid organs. These mice are characterized by grossly disrupted splenic compartmentalization and a lack of secondary lymphoid tissues (1,2). Lymph node agenesis may result from either faulty development of lymph node stromal support structures or defective lymphocyte localization to the developing lymph nodes. The specific role played by LT-α in the formation of lymph nodes and Peyer's patches (PP) is undefined beyond the essential involvement of the LT-beta receptor during embryogenesis. LT-α likely has little involvement in the development of lymphatic and blood vessels since lymphatic vessels have been used as markers to confirm failed node development and no reports of vascular agenesis are recorded in LT-α deficient mice (3,4). Because lymphocyte homing from blood into lymph nodes and PP is largely determined by interactions between "homing molecules" expressed on lymphocytes and "addressins" expressed on vascular endothelial cells, the phenotype of the LT-α deficient mouse could result from impaired expression of either of these complimentary molecules. L-selectin, the naïve lymphocyte homing molecule which permits entry to secondary lymphoid tissues, is reported to be expressed at equivalent levels in LT-α deficient mice and their wild-type litter-mates (1). Interestingly, these same LT-α deficient mice demonstrated leukocytosis (1). This situation is reminiscent of findings in certain leukocyte adhesion deficiencies (5) and suggestive of impaired vascular addressin expression. In fact, *aly,* LT-α, and tumor necrosis factor receptor Type I (TNFRI/p55) deficient mice lack splenic marginal zone

staining for the addressin, MAdCAM-1 (mucosal addressin cellular adhesion molecule), which is proposed to direct B cell segregation to splenic white pulp (6,7,8). MadCAM-1, the counter receptor for $\alpha 4\beta 7$ integrin expressed on activated lymphocytes, is selectively expressed on high endothelial venule endothelium of PP and within the gut lamina propria (LP) (9).

The relationship between splenic and gut expressed MAdCAM-1 is unknown. However, the lack of splenic expression of this addressin and the absence of gut associated lymphoid tissues in LT-α deficient mice led to speculation that impaired expression of specific vascular addressins or cellular homing molecules may contribute to the mutant phenotype. To test these hypotheses, the phenotypes of splenic lymphocytes from LT-α deficient and wild-type mice were examined by fluorescent activated cell scanning (FACS) analysis. Further, the presence of certain vascular endothelial cell addressins and lymphocyte homing molecules was investigated by immunohistochemistry. The data presented here suggest that impaired expression of select cell homing and vascular addressin molecules is not likely to be the cause of the lack of secondary and gut associated lymphoid tissues in adult LT-α deficient mice. Further, this presents novel information allowing further characterization of the LT-α deficient mouse and describes techniques for the immunohistochemical detection of lymphocyte and endothelial cell surface molecules in paraffin embedded tissues.

Chapter 3: Materials and Methods

Mice

Development and identification of mice rendered genetically deficient in LT-α (hereafter referred to as mutant) has been described previously (1). Original homozygous mutant female mice were bred to C57BL/6 males obtained from Jackson Laboratory (Bar Harbor, ME). All mutant mice used in the studies described here were the progeny of mutant or wild-type (hereafter referred to as w.t.) offspring from F8 heterozygous brother/sister interbreeding. Mice were terminated for cell and tissue analysis either at 8-10 weeks of age or were terminated from breeding at 18 months of age. A total of 8 mutant (5 young and 3 aged) and 7 w.t. (4 young and 3 aged) mice served as subjects for these studies. All mice were housed throughout the study in microisolator cages and maintained in a barrier facility at the Walters Life Sciences Building, University of Tennessee, Knoxville, and handled in accordance with institutional guidelines.

Sample Collection

Spleens were individually aseptically removed and portioned to allow for both gross fixation and single cell isolation. Red blood cell depleted suspensions of whole splenocytes were prepared to a concentration of 1.0.x 10^6 cells/ml in RPMI complete media (RPMI supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine). All populations were analyzed individually by FACS analysis.

Spleens, livers, lungs, mesentery (containing mesenteric lymph nodes (MLN) in w.t. mice), and all intestinal segments were removed from animals under sterile conditions and placed in periodate/lysine/paraformaldehyde (PLP) solution for storage at 4 °C for 24 hours. Samples were subsequently washed in 1x PBS, dehydrated, and infiltrated for embedding in low melting point (51-53 °C) paraffin as previously described (10). Serial 6 μm sections were cut on a microtome, placed on positively charged slides, and either stained with hematoxylin and eosin following standard techniques or stored at -20 °C until immunohistochemistry could be performed.

Cell Staining

Fluorescein isothiocyanate- (FITC) or phycoerythrin-(PE) conjugated antibodies against murine CD45RB/B220, Thy1.2, and L-selectin (Mel-14) (PharMingen, San Diego, CA) were incubated with splenocyte populations for 1 hour at 4 °C. Cells were washed three times with staining buffer (1% fetal bovine serum, 0.1% sodium azide, 1x PBS) and fixed in 1% paraformaldehyde (Sigma, St. Louis, MO). Samples were stained to simultaneously detect the following populations; Thy 1.2 vs. B220, L-selectin vs. Thy1.2 and L-selectin vs. B220. Samples were analyzed using a FACScan flow cytometer and Cell Quest software (Becton Dickinson, Mountain View, CA). A minimum of 10,000 events were analyzed per sample. Results presented are the mean of each group with appropriate standard errors displayed.

Immunohistochemistry

Slides with paraffin embedded tissue sections were deparaffinized in a xylene substitute (Fischer Scientific, Pittsburg, PA) (3 x 5 min), and rehydrated through decreasing concentrations of ethanol to distilled water at room temperature (RT). Endogenous peroxidase was blocked with 0.3% H_2O_2 for 15 minutes at RT. Sections were then washed in RT 1x PBS for 5 minutes and digested with 0.0025% trypsin in 0.1% $CaCl_2$, pH 7.8, at RT for 5 minutes. Slides were washed individually in a gentle stream of RT 1x PBS for 1 minute each. Non-specific protein binding was blocked by incubation of tissue sections in RT 10% heat inactivated goat serum for 30 minutes followed by addition of primary antibodies and incubation for 14-18 hours at 4 °C. Sections were washed with 1x PBS as described above, biotinylated secondary antibodies were applied and sections were incubated for 4 hours at RT. Following washing, avidin-biotin horseradish peroxidase (HRP) complex (ABC) (Vector, Burlingame, CA) was applied and incubated for 30 minutes at RT. The substrate for HRP, Vector VIP (Vector), was added and incubated at RT for 20 minutes. Tissue sections were counterstained with methyl green (Vector) and permanently cover slipped. **Table 1** (page 86) lists the primary antibodies used in this study.

Statistics

The significance of differences in cell populations were determined by Student's Two-tailed t-test where applicable. P values of less than 0.05 were considered significant.

Chapter 4: Results

Expression of lymphocyte surface molecules

LT-α deficient (mutant) mice lack all peripheral lymphoid tissues including MLN and PP. Curiously, these mice develop splenic hypertrophy with age and parenchymatous foci which resemble lymphoid tissues (1, 11). Since lymphocyte emigration to tissues largely depends on the expression (or lack thereof) of L-selectin, lymphocytes from mutant mice were expected to display alterations in the expression of this homing molecule. We tested this hypothesis by comparing lymphocyte phenotypes from mutant and w.t. mice by two-color FACS analysis. As displayed in Table 2 (page 87), expression of L-selectin by w.t. and mutant mice showed only minimal differences both between the groups and with age. Specifically, the co-expression of L-selectin with B220 or Thy1.2 decreased with age in both groups. Although the greatest percent decrease was most evident for the co-expression of L-selectin and Thy1.2 in aged mutant mice, there was no statistical significance to any changes observed. Keeping in mind that all mice were maintained in a barrier facility under sterile conditions and had no exposure to immunogens except those found in their sterilized feed, the decreased levels of L-selectin may indicate that mutant mice harbor higher populations of activated B and T cells than do w.t. mice. Perhaps mutant mice, in compensating for the lack of secondary lymphoid tissues, maintain higher populations of activated lymphocytes. This could explain the development of ectopic lymphoid tissues which develop in older mutant mice as described below and elsewhere (1,11).

Expression of vascular addressin and cell homing molecules

The expression of L-selectin on naïve lymphocytes permits entry to peripheral lymph nodes via binding to its counter-ligand, peripheral node addressin (PNAd) (12). Following cellular activation, there is an over-lapping but sequential down-regulation of L-selectin and an up-regulation of $\alpha4\beta7$ expression (13) during which time both L-selectin and $\alpha4\beta7$ interact with MAdCAM-1, possibly increasing the strength of attachment between lymphocyte and endothelial cell (13). $\alpha4\beta7$ mediates arrest of lymphocytes on MAdCAM-1 expressing endothelium, in an activation dependent fashion, thereby permitting entry to PP and lamina propria and subsequent effector functions (14). Blockage of either $\alpha4$ or $\beta7$ using monoclonal antibodies leads to near total abrogation of lymphocyte arrest and entry to lymphoid compartments (13). To determine the potential roles played by specific homing molecules and vascular addressins in the development of the mutant phenotype, MAdCAM-1, PNAd, and $\alpha4\beta7$, as well as the addressins ICAM-1 (intracellular adhesion molecule) and PECAM-1 (platelet/endothelial cell adhesion molecule) were detected in various tissues by immunohistochemistry. No differences in MAdCAM-1, ICAM-1, PNAd, PECAM-1, or $\beta7$ integrin staining was detected in any tissues from any mice, young or aged, w.t. or mutant. As expected and shown in **Figures 1a-d** (pages 72-74), positive staining for MAdCAM-1 was localized to small and medium sized venules and small arterioles located within the lamina propria of all intestinal segments (a-c), MLN (w.t. mice) (d), within mesenteric fat (mutant mice), and along the sinus lining cells of spleens.

Positive staining for PNAd, the carbohydrate ligand for L-selectin, was detected in spleens from both w.t. and mutant mice as well as MLN from w.t. mice (**Figures 2a-c**, pages 75-77). The expression of PECAM-1, an addressin which demonstrates constitutive expression on most endothelial cell surfaces, was faint to moderate in w.t. MLN and both mutant and w.t. spleens but present in all tissues examined (**Figures 3a-d**, pages 78-80).

$\alpha 4\beta 7$ was detected as a complex and by separate staining of its integrin components. All monoclonal antibodies allowed detection of positively stained cells within the spleens ($\alpha 4\beta 7$) and lamina propria ($\beta 7$) of both w.t. and mutant mice. The cells detected within intestinal villi presumably bore the $\alpha IEL\beta 7$ integrin complex (15) however expression of this complex was not specifically investigated. **Figures 4a-d** (pages 81-83) serve to illustrate such staining in spleens and intestinal sections.

ICAM-1 staining was faint but detectable along the endothelial lining of most small vessels in all intestinal sections and spleens examined (not shown). Since the up-regulated expression of this addressin is associated with inflammation, it's faint expression in mice included in this study, which were naïve to immunogens other than those contained in sterilized feed, was expected.

Chapter 5: Discussion

Lymphocyte emigration from the blood vasculature through high endothelial venules (HEV) to peripheral lymph nodes (PLN), MLN, and PP requires sequential and cooperative adhesion between lymphocyte surface-expressed homing molecules and endothelial cell surface vascular addressins (13, 16). Upon activation, lymphocytes destined to mucosal effector functions down regulate L-selectin expression and upregulate the integrin, $\alpha 4\beta 7$, which allows entry to mucosal PP and lamina propria via interactions with the mucosal vascular addressin, MAdCAM-1 (14). Monoclonal antibodies to these molecules function to completely abrogate lymphocyte entry to specific lymphoid tissue compartments and such *in vivo* manipulations have been used to determine the mechanisms involved in the cellular repopulation of lymph nodes (17). Mutant mice have normal levels of circulating lymphocytes yet develop no secondary or gut associated lymphoid tissue (1). These mice seem to have an opposite phenotype to severe combined immunodeficiency (*scid)* mice which have no circulating lymphocytes and empty lymphoid compartments (18). No lymphoid tissue anlages have been identified in mutant mice yet recreation of lymph nodes has been demonstrated following over-expression of the missing cytokine (3). Interestingly, these mice develop parenchymatous foci of mononuclear cells which have been described as ectopic lymphoid tissues (1,11). We have observed high percentages of IgG and IgM staining B cells as well as $CD4^+$ T cells in such foci (unpublished). Therefor, we questioned whether the mutant phenotype additionally involved alterations in lymphocyte homing and vascular addressin expression. We specifically investigated whether the expression

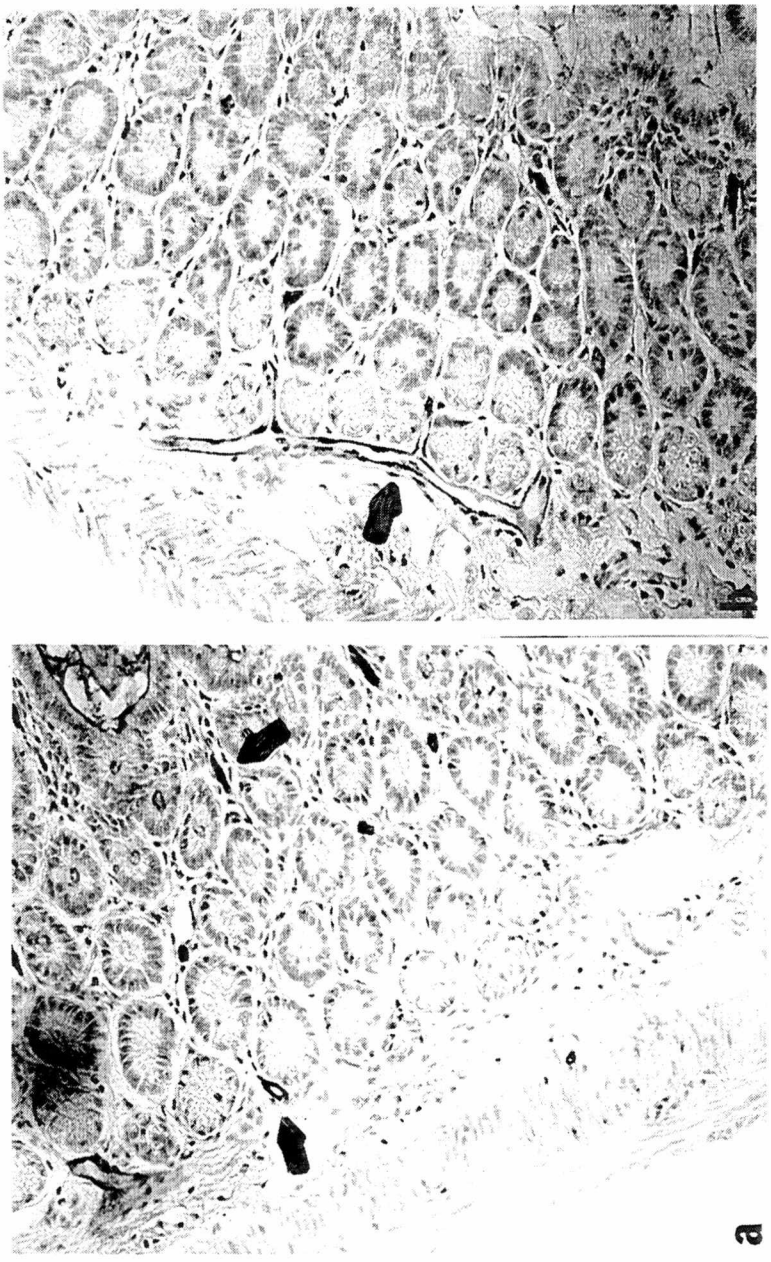
of lymphocyte homing molecules or vascular addressins differed between mutant and w.t. mice. We speculated that differences in expression of these molecules may contribute to the phenotype of the mutant mouse. However, we found no differences in expression of these molecules in any tissues examined by immunohistochemistry.

MAdCAM-1 expression was noted in the vasculature of all intestinal segments, all spleens, in PP and MLN from w.t. mice, and mesenteric fat from mutant mice. Similarly, positive staining for ICAM-1, PNAd, and PECAM-1 was equivalent in tissues from both mutant and w.t. mice. Cells staining positively for $\alpha 4\beta 7$ were primarily localized to intestinal villi and LP of all intestinal segments from all mice examined. Differences in lymphocyte expression of L-selectin did show variation, however, with aged mutant mice demonstrating lower lymphocyte expression than either young mutant mice or w.t. mice of any age. If the decrease in L-selectin expression in aged mutant mice is an indication of the level of lymphocyte activation, this may explain the development of ectopic lymphoid foci in these mice given that only activated lymphocytes can gain access to tissue sites. Further evidence that this may be the case is demonstrated in **Figure 5** (page 84-85), in which $\alpha 4\beta 7$ positive staining cells are detected in an ectopic pulmonary lymphoid foci of an aged mutant mouse. Although such foci, commonly seen in tissues of mutant mice over 12 weeks of age from our colony, may form in response to an inflammatory insult, the maintenance of these mice in a barrier facility under sterile conditions makes this unlikely. Conceivably, these foci represent a cell localization default pathway in a system where cells have limited organized structures to which they can home. Alternatively, these foci may represent a defect in the anti-inflammatory

effects which result from signaling through the tumor necrosis factor-α p55 receptor (TNFR1) (19). Specifically, TNFR1 deficient mice were found to be deficient in the production of manganese superoxide dismutase (MnSOD), a critical regulatory molecule which neutralized the reactive oxygen species released following triggering of the TNFR1. Without a means of checking the tissue damage associated with inflammatory conditions, cellular infiltration at inflammatory foci could proceed to result in severe cellular accumulations. Signaling through TNFR1 is not completely abrogated in LT-α deficient mice since TNF-α is present in (presumably) normal levels. Nevertheless, impaired MnSOD levels may contribute to the development of ectopic lymphoid foci.

The data presented here can be interpreted to mean that the phenotype of the adult mutant mouse may not include impaired lymphocyte migration to lymphoid tissues. Obviously, embryonic events have not been studied here with respect to cell homing events. However, although we found no significant differences in splenocyte phenotypes, a trend of decreased expression of L-selectin was observed as mutant mice aged. Therefor, adult mutant mice may compensate for their lack of secondary lymphoid tissues by maintaining elevated populations of activated lymphocytes which can selectively, and with some degree of organization, emigrate to tissues to form what appear to be functional lymphoid foci.

Figure 1: Detection of endothelial cells expressing the vascular addressin, MAdCAM-1, from wild-type (a and d) and mutant (b and c) small intestinal (a-c) or Peyer's patch (d) sections. All sections are magnified 50x. Positive staining is indicated by arrows. No difference is noted by visual inspection between wild-type or mutant tissues in the expression of MAdCAM-1.



a

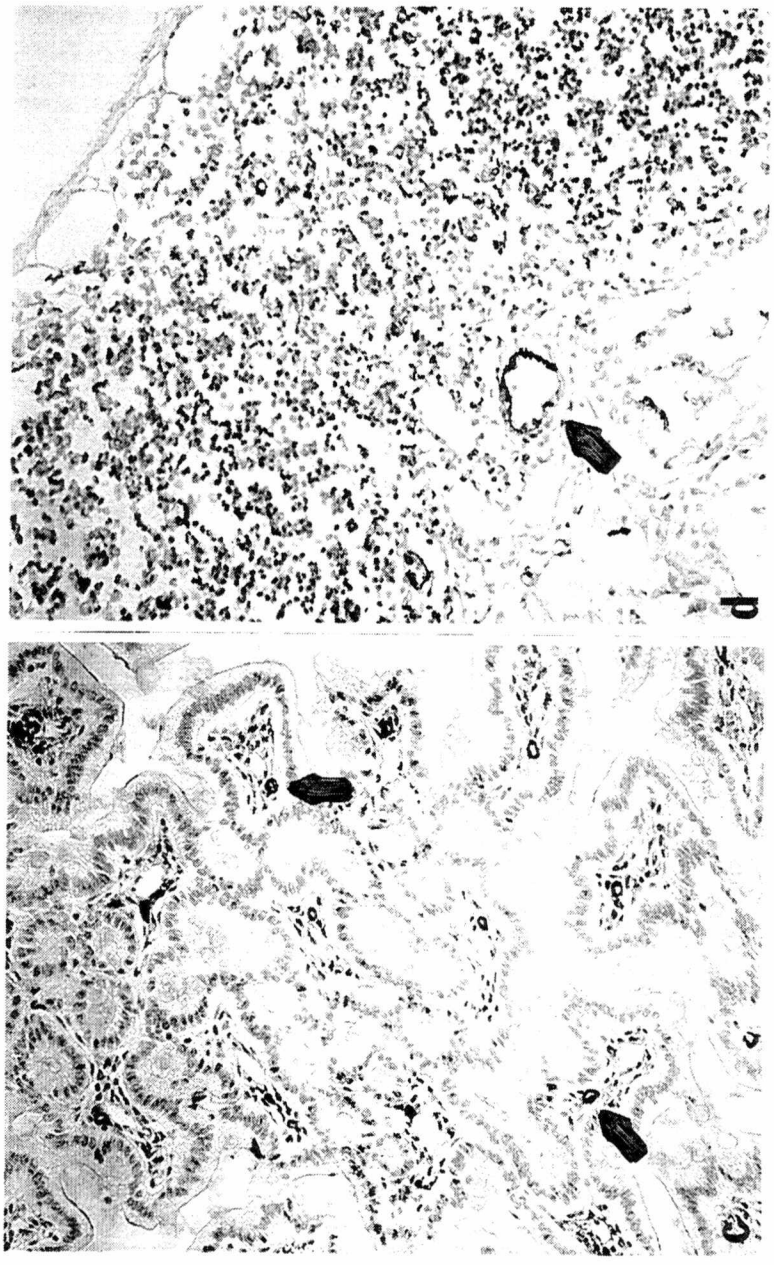
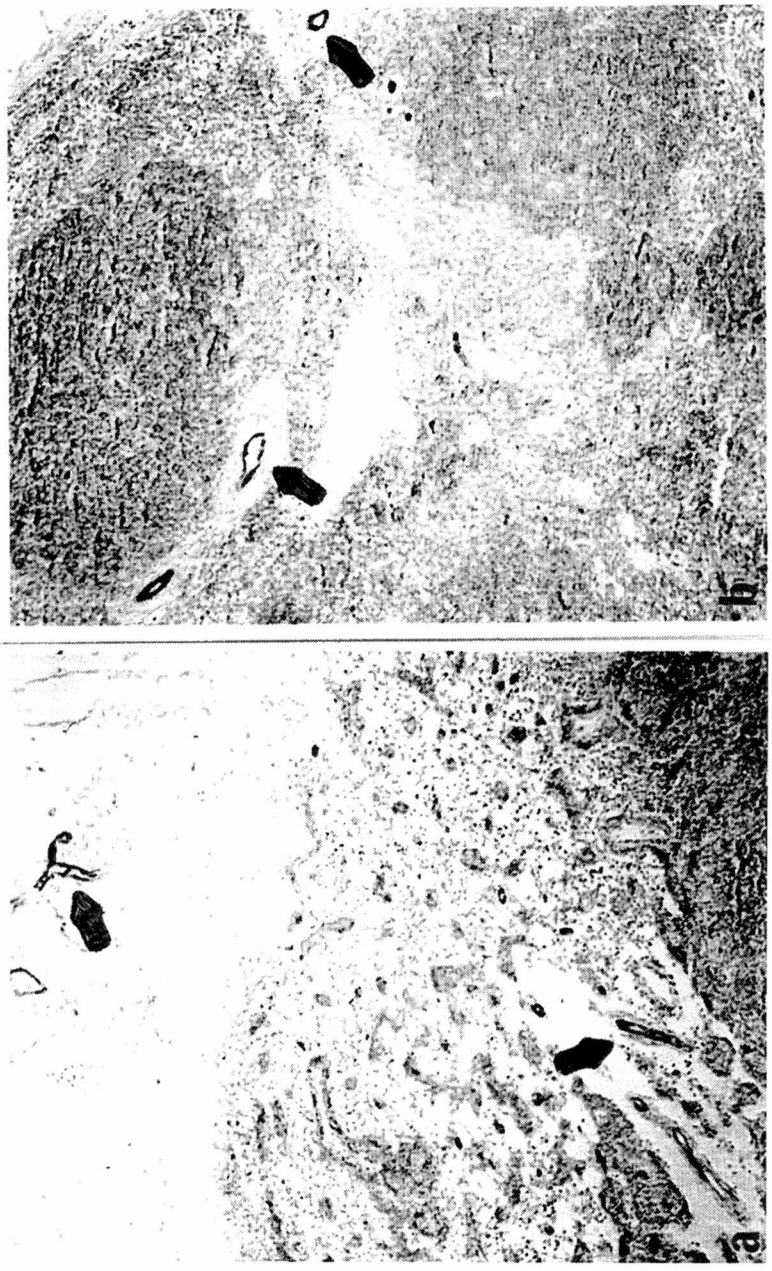


Figure 2: Detection of endothelial cells expressing the vascular addressin receptor for L-selectin, PNAd from MLN (a) or spleens (b and c) from wild-type (a and b) or mutant (c) mice. Magnification is 25x for 'a' and 'b' and 50x for 'c'. Positive staining is indicated by arrows.



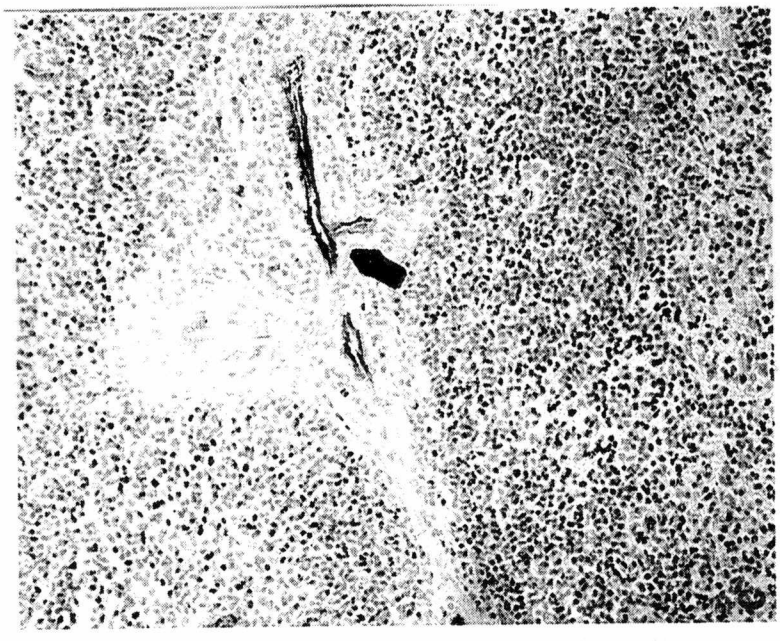
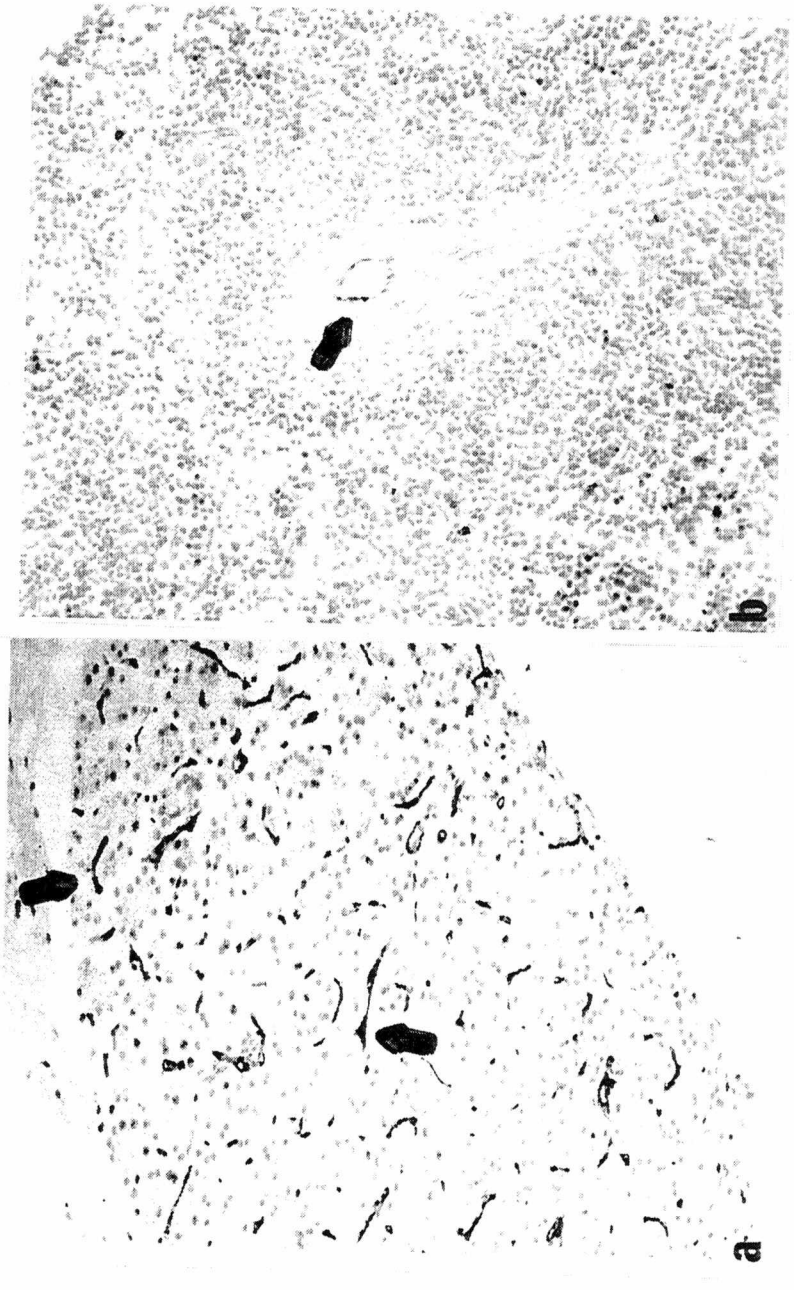


Figure 3: Detection of the vascular addressin, PECAM-1, in pancreas (a), spleen (b and c), and cecum (d) sections of wild-type (a and c) and mutant (b and d) mice. Section 'a' is used as the standard for positive staining, which is indicated in all figures by arrows. Note the foci of mononuclear cells present in section 'd'. Such foci, which are distinct from organized cecal lymph nodes, are typically found in sections from aged mutant mice. Magnification is 25x for 'a' and 50x for 'b-d'.



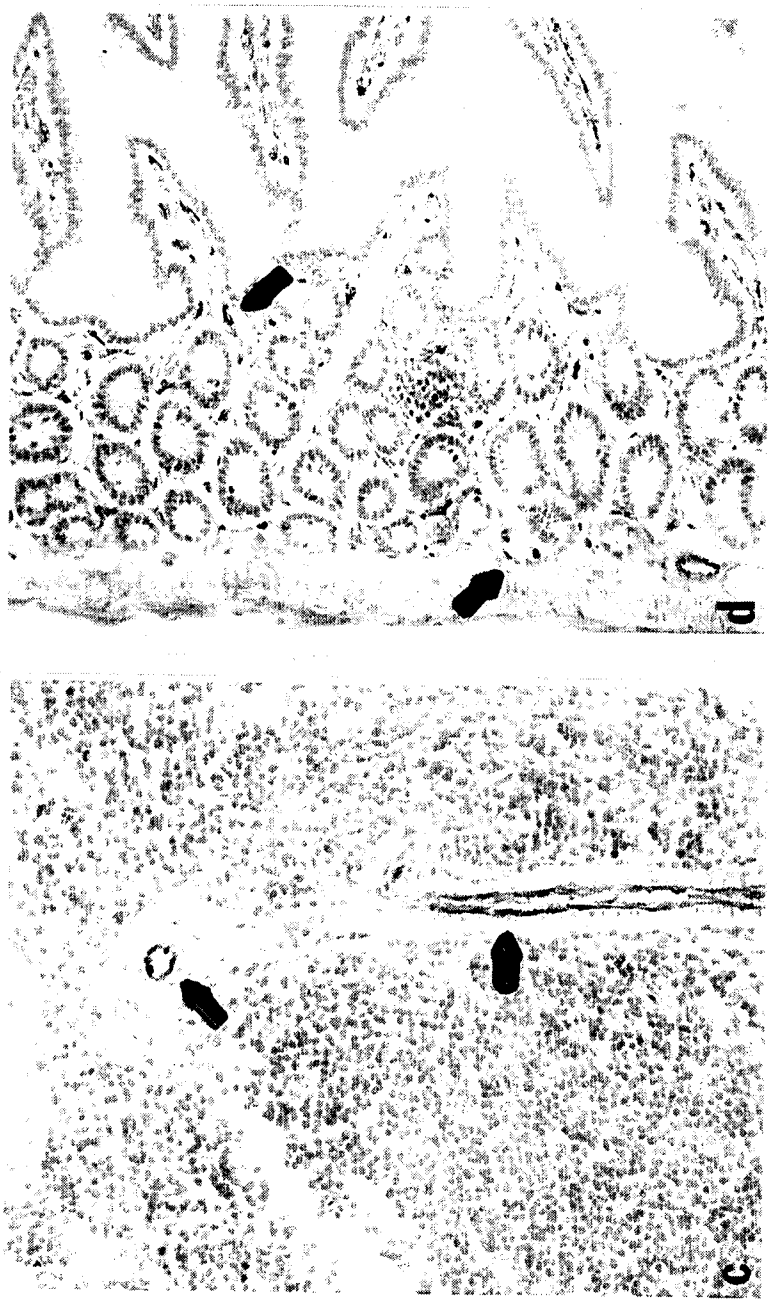
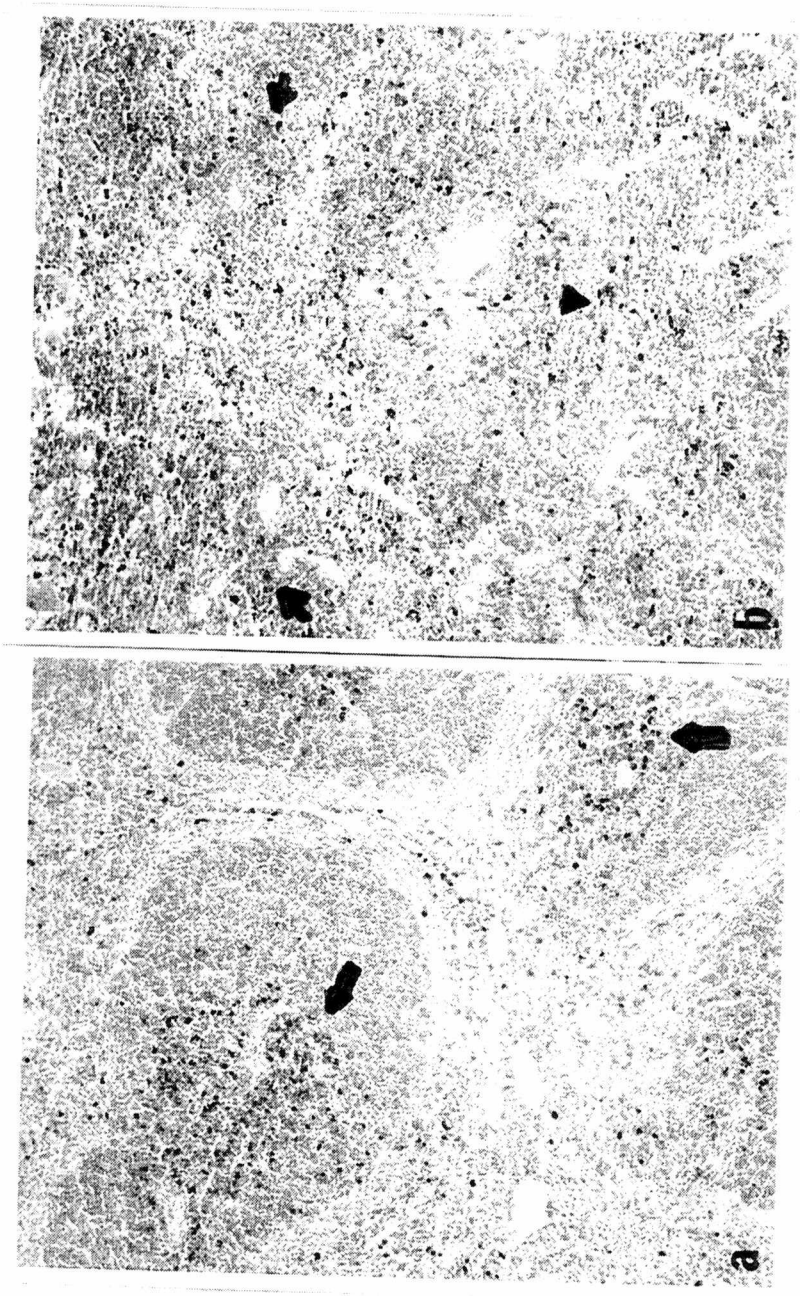
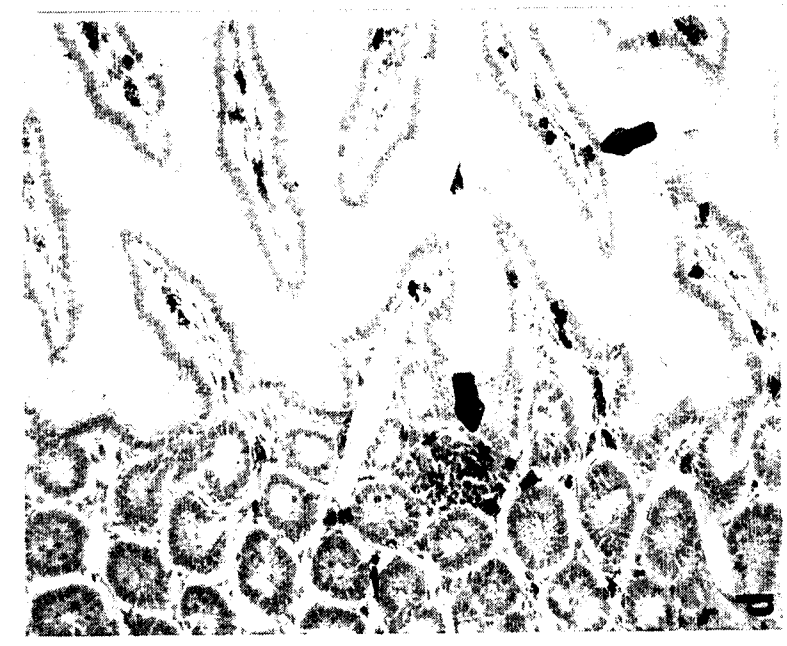

D
C

Figure 4: Detection of β7 integrin, a marker for activated lymphocytes, on mononuclear cells from spleen (a and b), small intestinal (c), and cecal (d) sections from wild-type (a) and mutant (b-d) mice. Splenic sections demonstrate the extremely disrupted architecture which characterizes the mutant mouse (a and b). Positively stained cells, indicated by arrows, are predominantly located within villi (c and d). Note the positively staining cells within the foci of mononuclear cells in the mutant cecal section (d). Spleens are magnified 25x and intestinal sections are magnified 50x.


b
a



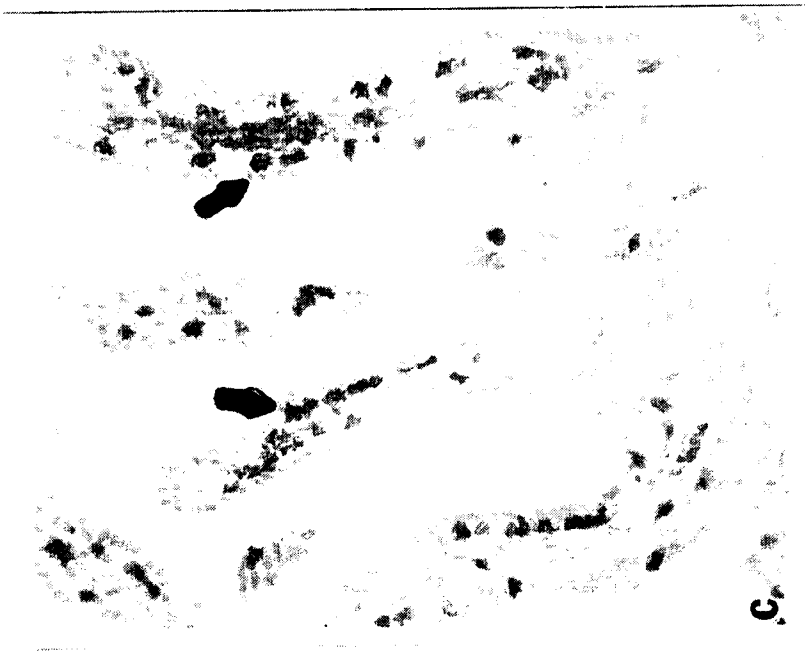


Figure 5: A section of lung from a retired mutant breeder displaying the typical perivascular accumulations of mononuclear cells seen in these mice. The cells indicated with arrows are staining positively for β7 integrin suggesting they are not naïve. Such foci may represent a degree of compensation by mutant mice for their lack of secondary lymphoid tissues. Magnification is 25x.

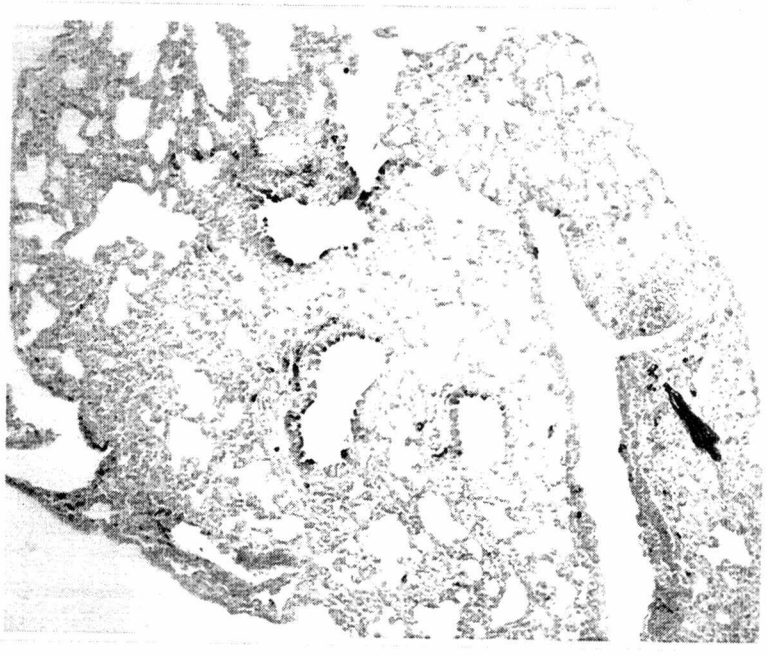


Table 1: Reagents used for immunohistochemical detection of vascular addressins and lymphocyte adhesion molecules. All antibodies were purchased from Pharmingen, San Diego, CA. All antibodies were purified and appropriate biotinylated secondary antibodies were used for detection.

Primary antibody	Clone	Dilution
Rat anti-mouse MADCAM-1	MECA-367	1:20
Rat anti-mouse L-selectin	MECA-79	1:20
Rat anti-mouse CD49d (α4 integrin)	R1-2	1:20
Hamster anti-mouse ICAM-1	3E2	1:10
Rat anti-mouse PECAM-1	MEC 13.3	1:50
Rat anti-mouse β_7 integrin	M293	1:40

Table 2: Phenotype of whole splenocyte populations from various mouse groups: Values presented the means of individual results and are expressed as percentages of the total cell population examined. Standard Error values are presented in parentheses below each value.

Group	B220	Thy1.2	B220 & L-Selectin	Thy1.2 & L-Selectin
Young w.t. spleen	46.0	28.5	31.7	18.4
(n=4)	(7.8)	(4.1)	(6.9)	(5.2)
Young mutant spln	51.4	30.2	23.1	14.8
(n=5)	(8.3)	(4.7)	(3.4)	(2.7)
Aged w.t. spleen	41.2	34.7	25.4	13.5
(n=3)	(6.8)	(5.9)	(4.8)	(5.1)
Aged mutant spln	53.2	31.5	18.5	8.2
(n=3)	(7.4)	(4.3)	(4.4)	(1.9)

Literature Cited

1. Banks, T. A., Rouse, B. T., Kerley, M. T., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D. M., Thomas, J., Kanangat, S., and Mucenski, M. L. 1995. Lymphotoxin-α-deficient mice: Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155: 1685-1693.

2. De Togni, P., Goellner, T., Ruddle, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. C., Carlson, R., Shornick, L. P., Strauss-Schoenberger, J., Russell, J. H., Karr, R., and Chaplin, D. D. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264: 703-707 .

3. Rennet, P. D., Browning, J. L., Mebius, R., Mackay, F., and Hochman, P. S. 1996. Surface lymphotoxin α/β complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184: 1999-2006.

4. Sacca, R., Turley, S., Soong, L., Mellman, I., and Ruddle, N. H. 1997. Transgenic expression of lymphotoxin restores lymph nodes to lymphotoxin-α deficient mice. *J. Immunol.* 159: 4252-4260.

5. Anderson, D. C. and Springer, T. A. 1990. Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1 and p150, 95 glycoproteins. *Ann. Rev. Med.* 38: 175-.

6. Tanaka, H., Hataba, Y., Saito, S., Fukushima, O., and Miyasaka, M. 1996. Phenotypic characteristics and significance of reticular meshwork surrounding splenic white pulp of mice. *J. Electron Microsc. Tokyo* 45(5): 407-416.

7. MacKay, F., Majeau, G. R., Lawton, P., Hochman, P. S., and Browning, J. L. 1997. Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice. *Eur. J. Immunol.* 27: 2033-2042.

8. Gonzales, M., Mackay, F., Browning, J. L., Kosco-Vilbois, M. H., and Noelle, R. J. 1998. The sequential role of lymphotoxin and B cells in the development of splenic follicles. *J. Exp. Med.* 187(7): 997-1007.

9. Briskin, N., Winsor-Hines, D., Shyjan, A., Cochran, N., Bloom, S., Wilson, J., McEvoy, L. M., Butcher, E. C., Kassam, N., Mackay, C. R., Newman, W., and Ringler, D. J. 1997. Human mucosal addressin cellular adhesion molecule is preferentially expressed in intestinal tract and associated lymphoid tissue. *Am. J. Path.* 151(1): 97-110.

10. Whiteland, J. L., Nicholls, S. M., Shimeld, C., Easty, D. L., Williams, N. A., and Hill, T. J. 1995. Immunohistochemical detection of T-cell subsets and other leukocytes in paraffin-embedded rat and mouse tissues with monoclonal antibodies. *J. Histochem. Cytochem.* 43(1): 313-320.

11. Alimzhanov, M. B., Kuprash, D. V., Kosco-Vilbois, M. H., Luz, A., Turetskaya, R. L., Tarakhovsky, A., Rajewsky, K., Nedospasov, S. A., and Pfeffer, K. 1997. Abnormal development of secondary lymphoid tissues in lymphotoxin β–deficient mice. *Proc. Natl. Acad. Sci. USA* 94: 9302-9307.

12. Streeter, P. R., Rouse, B. T. N., and Butcher, E. C. 1988. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biology* 107: 1853-1862.

13. Bargatze, R. F., Jutila, M. A., and Butcher, E. C. 1995. Distinct roles of L-selectin and integrins α4β7 and LFA-1 in lymphocyte homing to Peyer's patch- HEV in situ: the multistep model confirmed and redefined. *Immunity* 3: 99-108.

14. Berlin, C., Berg, E. L., Briskin, M. J., Andrews, D. P., Kilshaw, P. J., Holzman, B., Weissman, I. L., Hamann, A., and Butcher, E. C. 1993. α4β7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 74: 185-195.

15. Kilshaw, P. J. and Murant, S. J. 1991. Expression and regulation of β7 (βp) integrins on mouse lymphocytes: relevance to the mucosal immune system. *Eur. J. Immunol.* 21: 2591-2597.

16. Bradley, L. M., Watson, S. R., and Swain, S. L. 1994. Entry of naïve CD4 T cells into peripheral lymph nodes requires L-selectin. *J. Exp. Med.* 180: 2401-2406.

17. Tripp, R. A., Topham, D. J., Watson, S. R., and Doherty, P. C. 1997. Bone marrow can function as a lymphoid organ during a primary immune response under conditions of disrupted lymphocyte trafficking. *J. Immunol.* 158: 3716-3720.

18. Bosma, M. J. and Carroll, A. M. 1991. The scid mouse mutant: definition, characterization, and potential uses. *Annu. Rev. Immunol.* 9:323-350.

19. Castinos-Velez, E., Maerlan, S., Mosorio, L., Aberg, F., Biberfeld, P, Orn, A., and Rottenberg, M. C. 1998. *Trypanosoma cruzi* infection in tumor necrosis factor receptor p55-deficient mice. *Infect. Immun.* 66(6): 2960-2968.

Part 4: Immune responsiveness of lymphotoxin-alpha deficient mice: two reconstitution models.[1]

1. Submitted for publication to Cellular Immunology, manuscript number CI98-0126.

Chapter 1: Abstract

The effects of lymphotoxin–α (LT-α) deficiency on mucosal immune status has not been defined. We utilized severe combined immunodeficiency (*scid)* mice as recipients of both mutant and wild-type whole splenocytes to determine whether lymphocytes from mutant mice had impaired homing ability. We also utilized irradiated mutant mice as recipients of wild-type whole splenocytes to determine whether lymphoid tissue anlages had, indeed, failed to develop as a consequence of LT-α deficiency. Subsequently, all mice were immunized orally with an attenuated strain of *Salmonella typhimurium* and mucosal IgA responses were monitored. The data presented here demonstrate that *scid* recipients generate mucosal responses equally well when reconstituted with mutant or wild-type lymphocytes. In contrast, reconstitution of mutant mice with wild-type cells failed to affect the efficiency of their mucosal immunity. The mutant phenotype, therefor, appears to involve neither impaired lymphocyte homing nor function in the generation of mucosal immunity. However, the mutant phenotype and immune responsiveness cannot be transformed merely by the provision of LT-α expressing donor cell populations. The consequence of LT-α deficiency on mucosal immune responsiveness appears to be due to the lack of gut associated lymphoid tissues.

Chapter 2: Introduction

The phenotype of the lymphotoxin-alpha (LT-α) deficient (mutant) mouse leaves one to question how, in the often highly redundant immune system, can the absence of a single cytokine have such profound effects. A critical role for LT-α, via its interaction with surface bound lymphotoxin-beta (LT-β) at the LT-β receptor (LT-βR), in the development of secondary lymphoid tissue has been established (1, 2, 3, 4). This role was confirmed in separate experiments in which local LT-α expression in wild-type mice or ectopic expression of LT-α in LT-α deficient mice resulted in the development of mononuclear aggregations that morphologically resembled normal lymph nodes (5, 6).

LT–α also plays a critical role in the development of normal splenic architecture. Specifically, the initial reports of the LT-α gene-deleted mouse describe abnormally compartmentalized T- and B-cell zones within the splenic parenchyma but normal numbers of circulating B and T cells (1, 7). Further, reconstitution studies using severe combined immunodeficiency (*scid*) or sub-lethally irradiated LT-α wild-type (w.t.) or mutant mice as recipients confirmed that bone marrow or splenic lymphocytes from mutant mice were indeed capable of repopulating the existing lymphoid compartments of recipient mice (7 – 11). Several studies have investigated systemic immune responses of mutant and recipient mice to a number of parenterally administered antigens (4, 7-9, 11, 12). In most cases, strong IgM, but weak IgG, specific antigen responses were noted as well as a failure of formation of splenic germinal centers (GC) in recipients, regardless of the phenotype, when mutant donor cell populations were used.

Reports of mucosal immune responsiveness in LT-α deficient mice following oral antigen exposure are lacking. Impaired immune responsiveness in the mutant mouse is expected due to the absence of secondary and gut associated lymphoid tissue (GALT). However, whether this impaired responsiveness is due solely to the anatomical lack of lymphoid tissues or to an additional defect in lymphocyte function is unknown. To address these issues, we performed several reconstitution experiments using *scid* mice and irradiated mutant and wild-type mice as recipients of whole splenocytes from mutant and wild-type donors. Systemic and mucosal immune responsiveness to an orally administered attenuated strain of *Salmonella typhimurium* was monitored in the various study groups. We report the equivalent reconstituting ability of both mutant and w.t. whole splenocytes in *scid* recipients. However, the transfer of whole splenocytes from either w.t. or mutant mice failed to recreate GALT or any secondary lymphoid tissue structures in mutant recipients. Further, mucosal immune responses to the orally administered antigen were detected in all recipient mice but these responses appeared to be determined by the recipient and not the donor phenotype. Our data suggest that while lymphocytes from mutant mice are capable of homing to established lymphoid structures and compartments, the w.t. phenotype cannot be recreated in mutant mice merely by the transfer of w.t. splenocytes. Finally, we conclude that while necessary for optimal immune responsiveness, the presence of GALT is not essential for the development of specific mucosal immune responses following oral antigen administration.

Chapter 3: Materials and Methods

Mice

Development of mice rendered genetically deficient in LT-α has been described previously (7). These original homozygous mutant female mice were bred to C57BL/6 males obtained from the Jackson Laboratory (Bar Harbor, ME). Resulting heterozygous female offspring were bred back to their fathers for at least 6 generations. All mutant and w.t. mice used in the studies described here were the progeny of F5 heterozygous brother/sister interbreeding. Progeny mice were screened for the presence of the LT-α gene (w.t.) or the presence of the neomycin insert (mutant) by polymerase chain reaction (PCR) on prepared genomic DNA from tail tissue. C57BL/6 scid founder breeding mice were a kind gift from Dr. J. Russell Lindsey (University of Alabama, Birmingham). All mice were housed in microisolator cages and maintained in a barrier facility at the Walters Life Sciences Building, University of Tennessee, Knoxville, and handled in accordance with institutional guidelines.

Reconstitution of scid mice

These experiments were performed to determine whether whole splenocyte populations from mutant and w.t. mice were equally capable of reconstituting the empty lymphoid compartments of *scid* mice. *Scid* recipient mice between the ages of 6-8 weeks were lethally irradiated, receiving a total body radiation dose of 750 rads, 6 hours prior to injection of donor whole splenocytes. 1.0×10^7 donor mutant or w.t. whole splenocytes in 0.75 ml sterile 1x physiologic buffered saline (PBS) at room temperature (RT) were

administered to recipient mice intraperitoneally (IP). The IP route of administration was chosen based on preliminary work comparing the efficacy of intravenous versus IP delivery of different concentrations of whole splenocytes. To assess success of reconstitution, select recipients were euthanized two and four weeks post-reconstitution and tissues collected for fluorescent activated cell sorter (FACS) analysis or immunohistochemistry (IHC). For determination of immune responses to orally administered antigen and thus the functional state of reconstituting cell populations, recipient mice were immunized *per os* at 14 and 24 days post-reconstitution.

Reconstitution of mutant or w.t. mice

Mutant or w.t. recipient mice between the ages of 6-8 weeks were lethally irradiated, receiving 750 total body rads, 6 hours prior to injection of donor whole splenocytes. 1.0×10^7 donor mutant or w.t. whole splenocytes in 0.75 ml sterile 1x PBS at RT were administered to recipient mice IP. Mice were euthanized and/or immunized as described above.

Immunizations

Salmonella typhimurium strain BRD847, which expresses tetanus toxin, fragment C (ToxC), has been well characterized (13,14) and was used under agreement with Medeva PLC (Surrey, UK) to orally immunize mice in this study. Cultures were added to Luria-Bertani (LB) broth with 50 μg/ml ampicillin and incubated 16 hours at 37 °C. The culture was then diluted 1:50 in fresh LB broth with ampicillin and cultured for an

additional 4 hours at 37 °C. The bacteria were then pelleted at 6000 x *g* at 4 °C and resuspended in 5 ml ice cold 1x PBS. Mice were held off feed and water for 3 hours, placed under general inhalant anesthesia (Metofane, Mallinckrodt Veterinary, Inc., Mundelein, IL), and dosed orally with 100 µl (approximately 1.0 x 10^9 colony forming units (CFU)) of the bacterial cultures via an orogastric feeding needle. Bacterial cultures were serially diluted and plated on LB-ampicillin agar to confirm CFU given to mice.

Flow cytometry

Spleens were removed from animals under sterile conditions, divided to allow for individual histology and IHC, then pooled for each group for FACS analysis. Cells were isolated following gentle pressing of spleen segments through wire mesh screens into cold RPMI 1640 medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat inactivated fetal bovine serum (Gibco/BRL), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine. Red blood cells were lysed and whole spleen cells were adjusted to 1.0 x 10^6 cells/ml in FACS staining buffer (0.1% sodium azide and 5% fetal calf serum in PBS) following standard procedures. Fluorescein isothiocyanate-(FITC) or phycoerythrin-(PE) conjugated antibodies against murine CD4, CD8, CD45RB/B220, and Thy1.2 (PharMingen, San Diego, CA) were incubated with appropriate cell populations for 1 hour at 4 °C. Biotinylated PNA (Vector Laboratories, Inc., Burlingame, CA) was similarly incubated with cells followed by incubation with streptavidin-labeled FITC (PharMingen). Cells were washed with staining buffer between all incubations and finally fixed in 1% paraformaldehyde (Sigma, St. Louis, MO).

Samples were analyzed using a FACScan™ flow cytometer and Cell Quest software (Becton Dickinson, Mountain View, CA). A minimum of 10,000 events were analyzed per sample.

Histology

Following euthanasia, tissues were individually collected from mice and placed in periodate/lysine/paraformaldehyde (PLP) solution, stored at 4 °C for 24 hours, washed in 1x PBS, dehydrated, and infiltrated for embedding in low melting point (51-53 °C) paraffin as previously described (15). Serial 6 μm sections were cut on a microtome, placed on positively charged slides, and either stained with hematoxylin and eosin following standard techniques or stored at -20 °C until IHC could be performed.

Immunohistochemistry

Slides with paraffin embedded tissue sections were deparaffinized in a xylene substitute (Fischer Scientific, Pittsburg, PA) (2 x 5 min), and rehydrated through decreasing concentrations of RT ethanol to distilled water. Endogenous peroxidase was blocked with 0.3% H_2O_2 for 15 minutes at RT. Sections were then washed in RT 1x PBS for 5 minutes and digested with 0.0025% trypsin (Gibco/BRL) in 0.1% $CaCl_2$, pH 7.8, at RT for 5 minutes. Slides were washed individually in a gentle stream of RT 1x PBS for 1 minute each. Non-specific protein binding was blocked by incubation of tissue sections in RT 10% heat inactivated goat serum for 30 minutes. The blocking serum was blotted off, primary antibodies applied, and slides were incubated for 14-18 hours at 4 °C.

Sections were washed with 1x PBS as described above, secondary antibodies were applied and sections were incubated for 4 hours at RT. Following washing, avidin-biotin horseradish peroxidase (HRP) complex (ABC) were applied and incubated for 30 minutes at RT. Substrates for HRP included diaminobenzidene (BioGenex, San Ramon, CA) and Vector VIP (Vector). Tissue sections were counterstained with either hematoxylin (Biogenex) or methyl green (Vector) and permanently cover slipped (Fischer). In most instances, IHC staining followed the protocol for multiple antigen labeling described by Vector. Primary antibodies included anti-mouse CD_4, anti-mouse CD 90.2 (Thy1.2), biotinylated anti-mouse CD 45R/B220 (Pharmingen), biotinylated anti-mouse IgM (Jackson Immunochemicals, West Grove, *PA),* and biotinylated peanut agglutinin (Vector). Biotinylated rabbit anti-rat IgG polyclonal (Pharmingen) was used as a secondary where needed.

Serum and fecal immunoglobulin determination

Serum and fecal samples were collected from individual mice on the day of reconstitution (Day 0) and at 2 and 4 weeks post reconstitution. Serum samples were stored at –20 °C until assays were performed. Fecal samples were diluted in 1 ml 0.1% sodium azide in 1x PBS per 100 mg of feces and vortexed for 15 minutes. Fecal samples were then centrifuged for 8 minutes at 6000 R.P.M., supernatant collected and stored at –20 °C until enzyme-linked immunosorbent assays (ELISA) could be performed. ELISAs were performed using 96-well flat bottom plates (Immulon 4, Dynatech Laboratories, Inc., Chantilly, VA) coated with recombinant ToxC (Boehringer Mannheim,

Indianapolis, IN) at 4 μg/ml, or with anti-mouse IgM (Jackson), IgG, IgG_1, IgG_{2a} (Southern Biotechnology Assoc., Birmingham, AL) or IgA (Zymed) at 2 μg/ml overnight at 4 °C. Non-specific protein binding was blocked with 3.0% non-fat dry milk in 1x PBS for 2 hours at 37 °C. Plates were washed 3 times with 1x PBS containing 0.05% Tween-20 (Sigma) between all incubations. Standards of known concentration [IgM, 250 ng/ml (Sigma), IgG, 100 ng/ml (Southern Biotechnology), IgG_1 and IgG_{2a}, 250 ng/ml (Southern Biotechnology), and IgA, 100 ng/ml (Zymed)] and samples were added to plates and serially diluted two-fold in 1x PBS. Secondary antibodies included HRP-rabbit anti-mouse IgM (Zymed), biotin-goat anti-mouse IgA (Zymed), and HRP-goat anti-mouse IgG, IgG_1 and IgG_{2a} (Southern Biotechnology). Peroxidase-conjugated streptavidin (Jackson) was the tertiary antibody for IgA determination. Secondary and tertiary antibodies were incubated for 90 minutes at 37 °C. Plates were developed using ABTS [2,2' –azino-di- (3-ethylbenzthiazoline) sulfonic acid, Sigma] solution (4.4 mg ABTS in 20 ml 0.1 M citric acid plus 20 ml 0.1 M dibasic sodium phosphate and 4 μl 30% H_2O_2). All samples were run in duplicate and average absorbance was read at 405 λ on an automated plate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA) and titers determined using the SoftMax Pro (Molecular Devices) program. Titers are expressed as a group mean, in ng/ml or mg/ml, with appropriate standard errors displayed.

Statistics

The significance of differences in antibody levels and cell populations were determined by Student's Two-tailed t-test where applicable. P values of less than 0.05 were considered significant.

Chapter 4: Results

Reconstituting ability of mutant and w.t. whole splenocytes in scid or lethally irradiated recipient mice

In an attempt to determine the functional immune impairment associated with the LT-α mutant phenotype, mutant and w.t. whole splenocytes were used as donor cells in separate reconstitution models using lethally irradiated *scid*, mutant, or w.t. mice as recipients. **Figures 1a-d** (pages 108-110) show that spleens of all recipient mice had similar populations of B220, Thy1.2, $CD4^+$, and $CD8^+$ positive staining cells, as determined by FACS analysis, regardless of the reconstituting donor cell phenotype. Spleens were collected at 2 and 4 weeks post-reconstitution from all recipient groups to determine whether reconstituting cells persisted in their new environment. Both mutant and w.t. cells persisted at stable levels throughout the 4 week study period. These data indicate that splenocytes from mutant mice had homing ability to established lymphoid tissue similar to that of w.t. splenocytes. In addition, splenocytes which persisted over the 4 week study period were basically phenotypically identical whether from mutant or wild-type donors.

Reconstitution with w.t. cells does not correct the mutant phenotype

Assuming that the lack of secondary lymphoid tissues and disrupted splenic architecture characteristic of the mutant phenotype are due to a lack of LT-α expression, correction of this phenotype may occur following replacement of the missing cytokine. This approach was shown to be effective when LT-α was over-expressed in the presence

of an inflammatory focus (6). This was apparently not the case, however, when the source of LT-α was from w.t. lymphocytes in whole splenocyte populations. The presence of the LT-α gene was confirmed by PCR in the reconstituted spleens of lethally irradiated mutant mice given w.t. cells (See Part 2, Chapter 2, Figure 1 for representative PCR data). However, no functional assays were performed to confirm the presence of the LT-α gene product in these reconstituted tissues. Nevertheless, in no situation in our studies was the mutant phenotype transformed to a w.t. phenotype. The splenic architecture of mutant recipients remained disorganized with respect to B and T cell areas, regardless of the reconstituting cell population (**Figures 2a-2f**, pages 111-112). Further, despite the provision of the missing cytokine in certain donor cell populations, no secondary lymphoid tissues or GALT structures were generated *de novo* in any reconstituted mutant mice. However, mutant donor cells were capable of repopulating not only the empty lymphoid compartments of *scid* mice but all lymphoid compartments examined in w.t. recipients (data not shown). In conclusion, in the models presented here, the homing ability of mutant lymphocytes appeared unimpaired and the mutant phenotype is seemingly a non-reversible embryonic defect.

Mucosal immune responses are determined by recipient phenotype, not donor cell genotype

DeTogni and Banks (1,7) independently reported little difference in the levels of total serum IgM and IgG as well as similar numbers of circulating lymphocytes between mutant and w.t. mice. We also compared the levels of total serum IgG in mutant and w.t.

mice with levels in the various reconstitution groups and found minimal difference (differences were not statistically significant) (**Figure 3**, pages 113-114). The ability of reconstituting donor cells of both the mutant and w.t. phenotypes to home and persist within recipient spleens and contribute to systemic immunoglobulin levels is therefor equivalent. In contrast, however, this scenario did not hold true when comparing mucosal immune function as assessed by total fecal IgA levels between the various reconstituted groups. As shown in **Figure 4** (pages 115-116), a dramatic difference in levels of fecal IgA was evident between mutant and w.t. recipients and this was unaltered following reconstitution with either genotypic donor population. Mutant and w.t. whole splenocytes were, however, equally capable of reconstituting the empty GALT compartments of *scid* mice as evidenced by similar levels of total IgA in these groups. The inability of any donor splenocyte population to elevate the mucosal immune responsiveness of mutant recipients to that of w.t. recipients is further demonstrated in **Figure 5** (pages 117-118) which records ToxC specific IgA responses. Mutant recipients, although demonstrating no regeneration of GALT or secondary lymphoid tissue components, were capable of mounting limited antigen specific mucosal immune responses. These responses were, however, significantly reduced from those of w.t. or *scid* recipients, regardless of the genotype of the donor cell source. The phenotype of the recipient, therefor, appears to dictate mucosal immune responsiveness quantitatively. The superior responsiveness of w.t. and *scid* recipients is suspected to be related to the presence of organized GALT components, namely, PP and MLN.

Chapter 5: Discussion

Reports of mucosal immune responsiveness in the LT-α deficient mouse are lacking. Severe impairment in mucosal immune responsiveness was the expected consequence in this mouse strain due to the lack of all secondary and gut associated lymphoid tissues. We have attempted to demonstrate the immune function capacity of the LT-α deficient mouse in separate reconstitution studies designed to dissect the contribution of established lymphoid tissues from that of lymphocytes to mucosal immune responses. We have confirmed that mutant lymphocytes are not impaired in their homing ability and persist in reconstituted recipient spleens throughout our 4 week study period. Further, when established lymphoid tissues are present, reconstituting mutant whole splenocytes function to generate specific mucosal antibody responses equivalent to those of w.t. cells. However, in no situation could any donor cell population correct the splenic architecture or regenerate GALT structures in mutant recipients. Similarly, mutant recipients were incapable of generating mucosal immune responses equivalent to those of either w.t. or *scid* recipients regardless of the reconstituting donor cell genotype. The phenotype of recipient mice, therefor, appears to be critical for the subsequent generation of mucosal immune responses following oral administration of attenuated *Salmonella typhimurium*.

Previous studies in mutant mice used parenterally administered sheep red blood cells (SRBC), nitrophenol (NP)-haptenated chicken γ globulin in alum, NP-ovalbumin in alum, or KLH in incomplete Freund's adjuvant to generate immune responses (4,7-9, 11, 12). In most cases, strong IgM (but weak IgG) responses were detected specific to the

administered antigen indicating appropriate antigen delivery to functionally competent B-lymphocytes. These studies focused on the absence of critical lymphoid follicle structures, namely follicular dendritic cells and failed germinal center formation, to explain the impaired systemic immune responsiveness of mutant mice. While the spleen may be important for the generation of systemic immune responses, gut associated lymphoid tissues are deemed critical for the generation of mucosal immune responses to orally administered antigen (16). The LT-α deficient mouse provides a good model for determining the validity of this viewpoint. *S. typhimurium* strain BRD847 was chosen as an oral antigen because generated immune responses are T dependent (14), it selectively gains entry through M cells (13), and is not lethal following oral administration, even in *scid* mice.

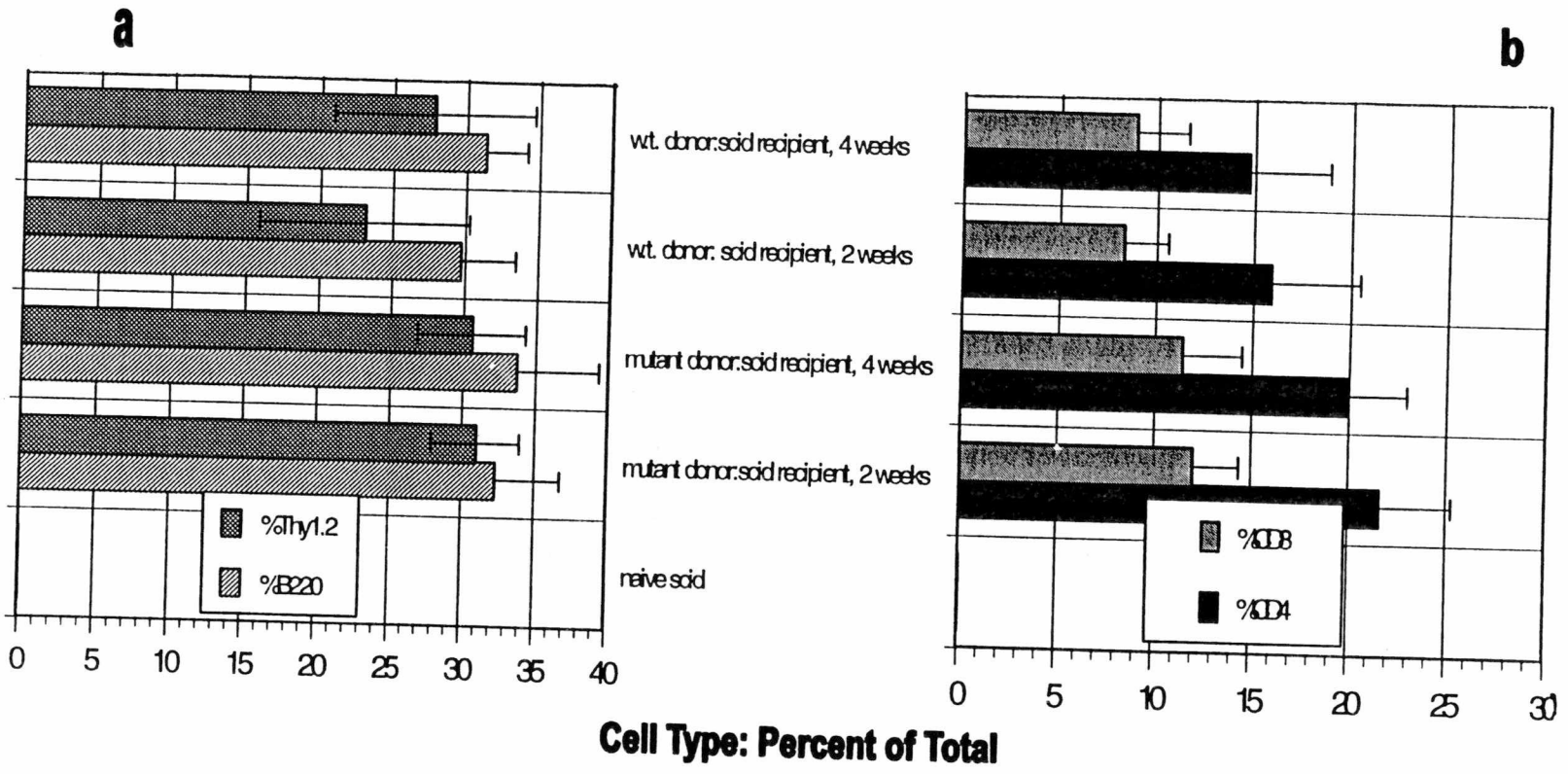
Examination of surface markers of lymphocytes recovered from reconstituted spleens at 2 and 4 weeks post-transfer revealed little difference in population phenotype regardless of donor or recipient population. Indeed, T and B cells from both mutant and w.t. mice appeared to persist equally well in all recipient groups as demonstrated by both FACS analysis and serum IgG levels. While a portion of the measured IgG in mutant and w.t. recipients could have been residual, given that irradiation does not destroy serum immunoglobulins, the maintenance and elevation of serum IgG levels following oral administration of *S. typhimurium* strain BRD847 in most recipient groups indicates that the transferred lymphocytes played a dominant role in the production of the detected IgG levels.

In contrast, IgA levels in fecal slurries from variously immunized and reconstituted groups showed very distinct patterns. Preliminary studies using BRD847 orally in mutant and w.t. mice confirmed that mutant mice were indeed capable of mounting specific systemic and mucosal immune responses, albeit significantly less than those observed in w.t. mice (see Part 2). Generated total IgA and ToxC specific fecal IgA levels showed a definite dependence on recipient phenotype but not donor cell phenotype. Thus there appears to be a strong correlation between the presence of GALT and the ability to generate significant mucosal IgA. Mutant mice lack the PP and MLN mucosal inductive tissues, but seem to have normal intraepithelial (IEL) and lamina propria lymphocyte (LPL) cell populations (See Part 2). While the IEL and LPL are considered to harbor effector cell populations in w.t. mice (17), cells with these compartments may additionally serve inductive functions in mutant mice. Although BRD847 selectively enters via M cells, the specialized epithelium overlying PP, this organism may also enter through mucosal epithelial cells (13,17). Mucosal epithelial cells, in covering the entire surface of the GI tract, may function as antigen presenting cells, serving as inducers of immune responses in IEL and LPL T cells (16). In fact, although we have no direct evidence, it may be that the mucosal epithelial cells of the mutant mouse compensates for the missing PP and MLN inductive sites thereby allowing mutant mice to develop quantitatively impaired, but specific, mucosal immune responses following oral antigen administration. Regardless of the nature of the compensatory mechanisms in mutant mice which permit the generation of mucosal immune responses, fecal IgA production could not be significantly augmented in mutant recipients by

reconstitution with w.t. cells. However, the reconstitution of *scid* recipients with mutant cells significantly increased mucosal IgA production. Therefor, optimal mucosal IgA production, unlike systemic IgG production, shows a distinct requirement for the presence of organized secondary lymphoid tissues following oral antigen administration.

The work presented here supports that of others in confirming the unimpaired homing ability and functional capacity of lymphocytes from LT-α deficient mice. We add that these mutant mice are impaired in the generation of mucosal IgA responses following oral antigen administration and that this impairment is due to the lack of GALT. Further, we conclude that the mutant phenotype cannot be transformed to w.t., neither with respect to organized splenic architecture nor in the genesis of secondary lymphoid tissues, merely by the addition of w.t. lymphocytes possessing the LT-α gene. However, based on our findings of diminished but detectable mucosal immune responses in GALT deficient mutant recipients, we suggest that the immune compensatory ability of the mutant mouse extends to mucosal inductive pathways.

Figure 1: Results of FACS analysis of various recipient groups at 2 and 4 weeks post-reconstitution. Figures "a" and "c" demonstrate the percent of cells expressing B220 or Thy1.2 while figures "b" and "d" demonstrate cells expressing CD_4 or CD_8. No significant differences were noted in cell phenotype from any donor population.


a
b
wt. donor:scid recipient, 4 weeks
wt. donor: scid recipient, 2 weeks
mutant donor:scid recipient, 4 weeks
mutant donor:scid recipient, 2 weeks
naive scid
%Thy1.2
%B220
%CD8
%CD4
0 5 10 15 20 25 30 35 40
0 5 10 15 20 25 30
Cell Type: Percent of Total

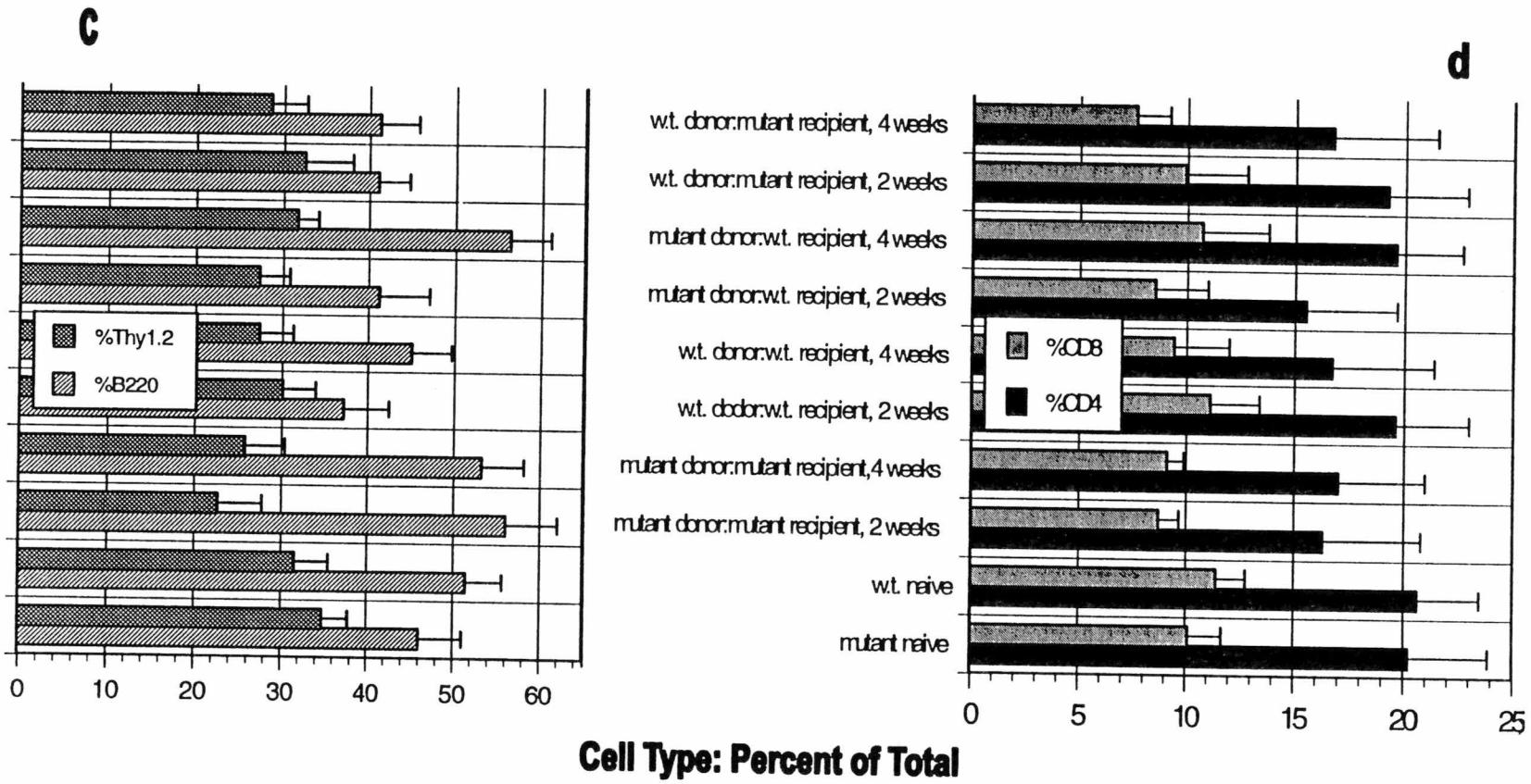

c
%Thy1.2
%B220
0
10
20
30
40
50
60
d
w.t. donor:mutant recipient, 4 weeks
w.t. donor:mutant recipient, 2 weeks
mutant donor:w.t. recipient, 4 weeks
mutant donor:w.t. recipient, 2 weeks
w.t. donor:w.t. recipient, 4 weeks
w.t. dodor:w.t. recipient, 2 weeks
mutant donor:mutant recipient,4 weeks
mutant donor:mutant recipient, 2 weeks
w.t. naive
mutant naive
%CD8
%CD4
0
5
10
15
20
25
Cell Type: Percent of Total

Figure 2: Immunohistochemical identification of recipient splenic B and T populations. H and E (a,c,e) or CD_4-DAB versus B220-VIP with hematoxylin counterstain (b,d,f) of spleens are displayed from the following experimental groups; "a" and "b"- wild-type, "c" and "d"- mutant recipient reconstituted with mutant donor cells, "e" and "f"- mutant recipient reconstituted with wild-type donor cells. CD_4^+ cells are brown, $B220^+$ cells are blue/purple and negatively staining cells are light blue. All figures are magnified 20X. In no case was the mutant phenotype transferred to the wild-type phenotype.

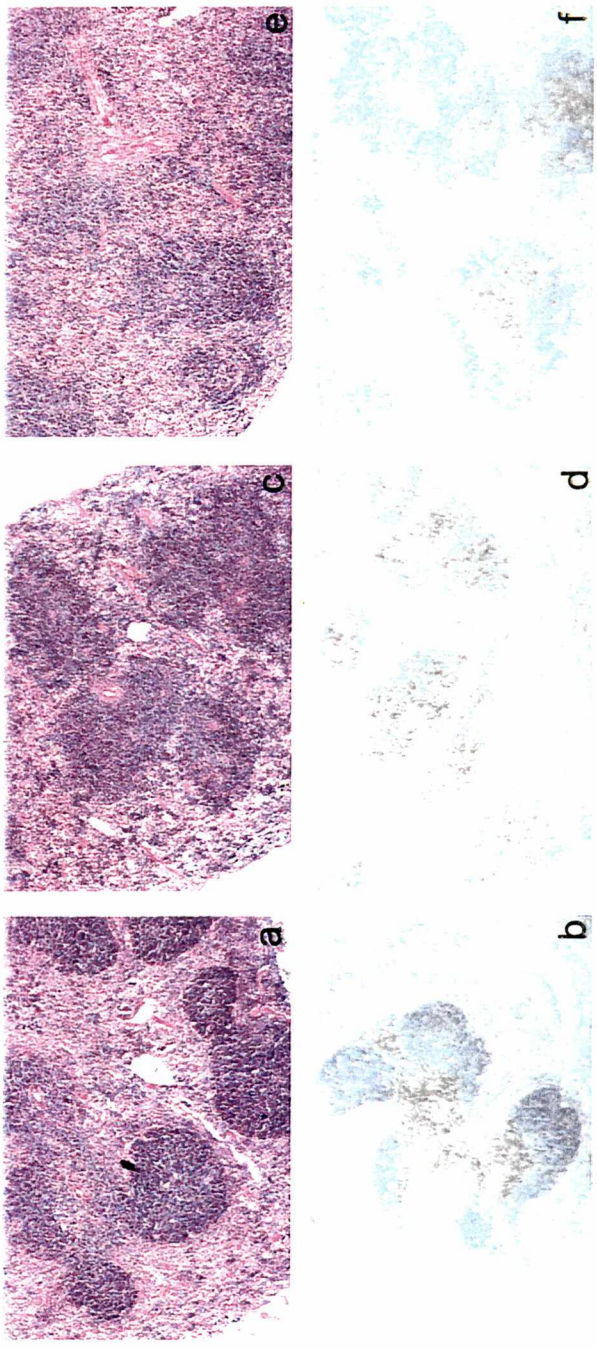

a
b
c
d
e
f

Figure 3: Total serum IgG levels in various recipient mice. Data presented are the means of total serum IgG levels detected from individual recipient mice at the indicated time points post-reconstitution. Mutant and wild-type donor cell populations were capable of generating similar serum IgG levels among the different recipient populations. The enhanced responses at 4 weeks were most likely in response to oral immunization. Levels are expressed in micrograms/ml of serum.

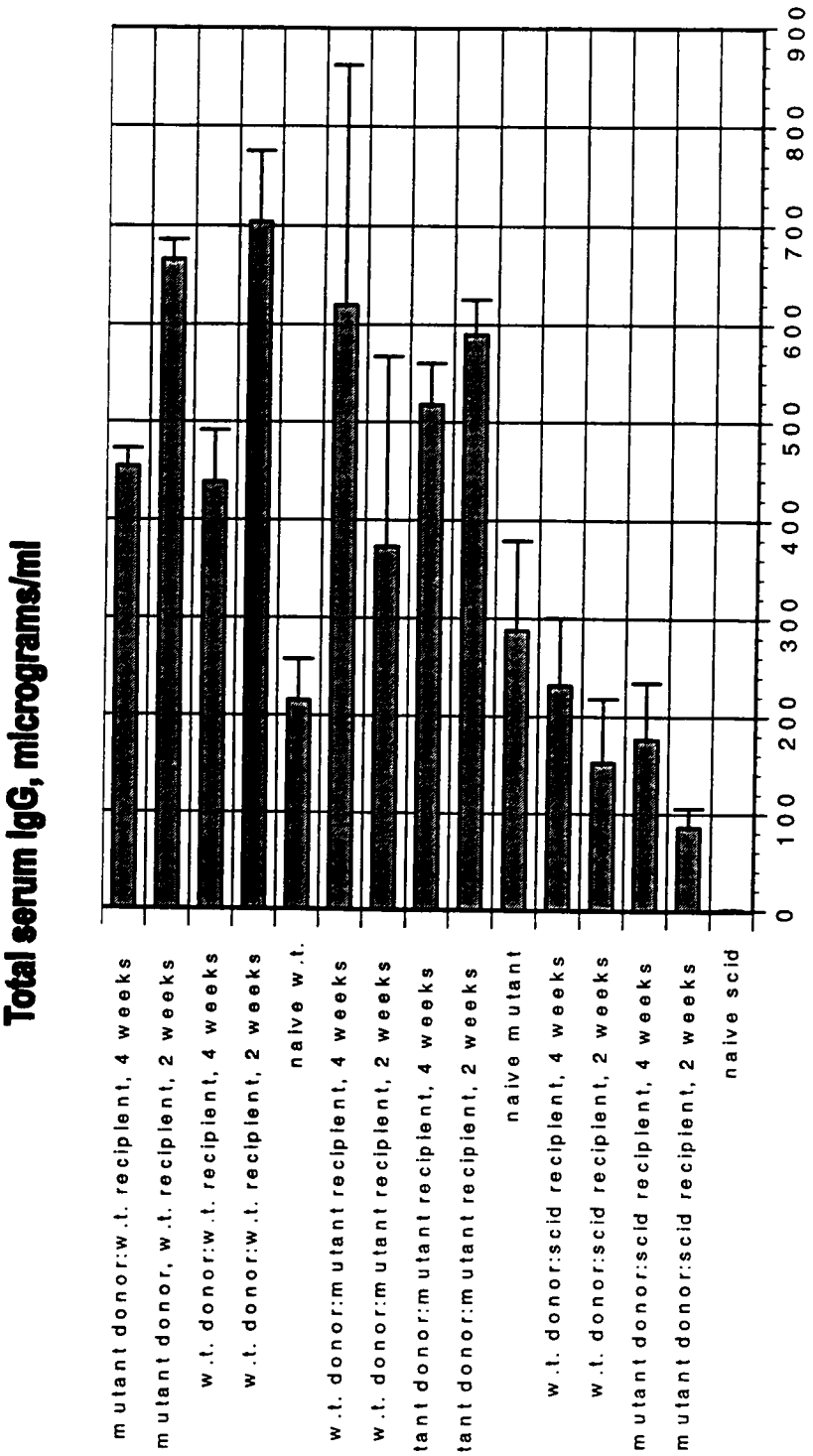

Total serum IgG, micrograms/ml
0
100
200
300
400
500
600
700
800
900
mutant donor:w.t. recipient, 4 weeks
mutant donor, w.t. recipient, 2 weeks
w.t. donor:w.t. recipient, 4 weeks
w.t. donor:w.t. recipient, 2 weeks
naive w.t.
w.t. donor:mutant recipient, 4 weeks
w.t. donor:mutant recipient, 2 weeks
mutant donor:mutant recipient, 4 weeks
mutant donor:mutant recipient, 2 weeks
naive mutant
w.t. donor:scid recipient, 4 weeks
w.t. donor:scid recipient, 2 weeks
mutant donor:scid recipient, 4 weeks
mutant donor:scid recipient, 2 weeks
naive scid

Figure 4: Total fecal IgA responses of various recipient mice. Comparisons of total fecal IgA responses from various reconstitution groups following oral *S. typhimurium* strain BRD847 administration measured in micrograms/ml of fecal slurry. Enhanced responses are seen in *scid* and wild-type recipients regardless of the donor cell genotype following antigen administration. Mutant recipients had severely impaired IgA responses regardless of donor cell genotype.

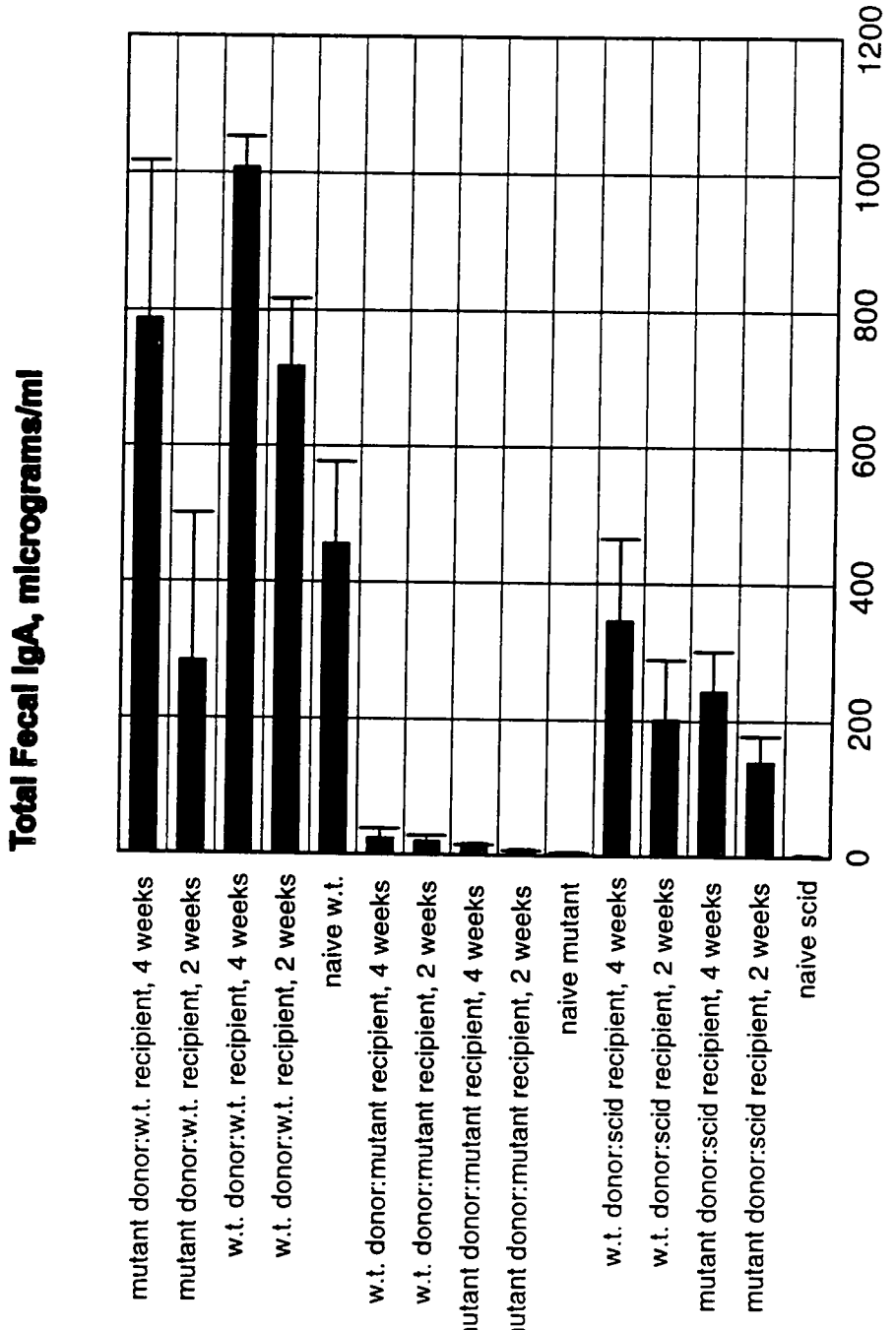
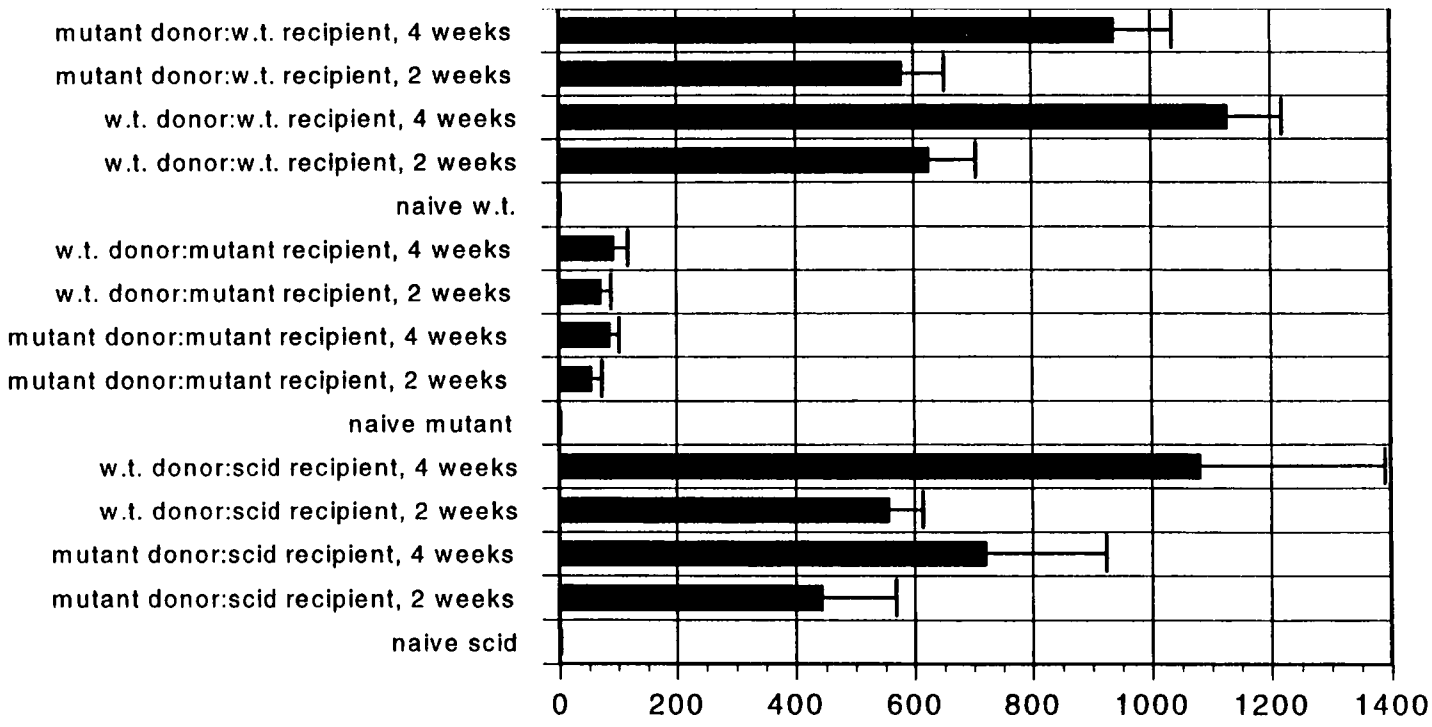

Total Fecal IgA, micrograms/ml
0
200
400
600
800
1000
1200
mutant donor:w.t. recipient, 4 weeks
mutant donor:w.t. recipient, 2 weeks
w.t. donor:w.t. recipient, 4 weeks
w.t. donor:w.t. recipient, 2 weeks
naive w.t.
w.t. donor:mutant recipient, 4 weeks
w.t. donor:mutant recipient, 2 weeks
mutant donor:mutant recipient, 4 weeks
mutant donor:mutant recipient, 2 weeks
naive mutant
w.t. donor:scid recipient, 4 weeks
w.t. donor:scid recipient, 2 weeks
mutant donor:scid recipient, 4 weeks
mutant donor:scid recipient, 2 weeks
naive scid

Figure 5: Comparisons of ToxC specific fecal IgA responses from various reconstitution groups following oral *S. typhimurium* strain BRD847 administration measured in nanograms/ml of fecal slurry. Responses mimic those of total IgA and indicate that mucosal responsiveness to orally administered *S. typhimurium* strain BRD847 is recipient phenotype dependent.

ToxC Specific Fecal IgA, ng/ml



Literature Cited

1. De Togni, P., Goellner T., Ruddle, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. C., Carlson, R., Shornick, L. P., Strauss-Schoenberger, J., Russell, J. H., Karr, R., and Chaplin, D. D. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264: 703-707.
2. Rennert, P. D., Browning, J. L., Mebius, R., Mackay, F., and Hochman, P. S. 1996. Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184: 1999-2006.
3. Ettinger, R., Browning, J. L., Michie, S. A., van Ewijk, W., and McDevitt, H. O. 1996. Disrupted splenic architecture, but normal lymph node development in mice expressing a soluble lymphotoxin-β receptor-IgG_1 fusion protein. *Proc. Natl. Acad. Sci. USA* 93: 13102-13107.
4. Koni, P. A., Sacca, R., Lawton, P., Browning, J. L., Ruddle, N. H., and Flavell, R. A. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. *Immunity* 6: 491-500.
5. Kratx, A., Campos-Neto, A., Hanson, M. S., and Ruddle, N. H. 1996. Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J. Exp. Med.* 183: 1461-1472.
6. Sacca, R., Turley, S., Soong, L., Mellman, I., and Ruddle, N. H. 1997. Transgenic expression of lymphotoxin restores lymph nodes to lymphotoxin-α-deficient mice. *J. Immunol.* 159: 4252-4260.

7. Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D. M., Thomas, J., Kanangat, S., and Mucenski, M. L. 1995. Lymphotoxin-α-deficient mice: effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155: 1685-1693.

8. Fu, Y.-X., Mohna, H., Matsumoto, M., Huang, G., Min, J., and Chaplin, D. D. 1997. Lymphotoxin-α (LTα) supports development of splenic follicular structure that is required for IgG responses. *J. Exp. Med.* 185 (12): 2111-2120.

9. Matsumoto, M., Mariathasan, S., Nahm, M. H., Baranyay, F., Peschon, J. J., and Chaplin, D. D. 1996. Role of lymphotoxin and the Type 1 TNF receptor in the formation of germinal centers. *Science* 271: 1289-1291.

10. Matsumoto, M., Fu, Y.-X., Molina, H., and Chaplin, D. D. 1997. Lymphotoxin-α-deficient mice and TNF receptor-1-deficient mice define developmental and functional characteristics of germinal centers. *Immunol. Rev.* 156: 137-144.

11. Matsumoto, M., Fu, Y.-X., Molina, H., Huang, G., Kim, J., Thomas, D. A., Nahm, M. H., and Chaplin, D. D. Distinct roles of lymphotoxin α and Type 1 tumor necrosis factor (TNF) receptor in the establishment of follicular dendritic cells from non-bone marrow-derived cells. *J. Exp. Med.* 186(12): 1997-2004.

12. Liu, Y.-J. and Arpin, C. 1997. Germinal center development. *Immunol. Rev.* 156: 111-126.

13. Chatfield, S. N., Charles, I. G., Makoff, A. J., Oxer, M. D., Dougan, G., Pickard, D., Slater, D., and Fairweather, N. F. 1992. Use of the *nirB* promoter to direct the stable

expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. *Bio/Technology* 10: 888-892.

14. Karem, K. L., Chatfield, S., Kuklin, N., and Rouse, B. T. 1995. Differential induction of carrier antigen-specific immunity by *Salmonella typhimurium* live-vaccine strains after single mucosal or intravenous immunizations of BALB/c mice. *Inf. Imm.* 63(12): 45574563.
15. Whiteland, J. L., Nicholls, S. M., Shimeld, C., Easty, D. L., Williams, N. A., and Hill, T. J. 1995. Immunohistochemical detection of T-cell subsets and other leukocytes in paraffin-embedded rat and mouse tissues with monoclonal antibodies. *J. Histochem. Cytochem.* 43(1): 313-320.
16. Mowat, A. M., and Viney, J. L. 1997. The anatomical basis of intestinal immunity. *Immunol. Rev.* 156: 145-166.
17. Jones, B. D., Ghori, N., and Falkow, S. 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized M cells of the Peyer's patches. *J. Exp. Med.* 180: 15-23.
18. Kato, T., and Owen, R. L. Structure and function of intestinal mucosal epithelium. *In:* Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGhee, J. R., and Bienenstock, J., eds. Handbook of Mucosal Immunology. San Diego: Academic Press Inc., pp: 11-26.

Part 5: Effect of splenectomy on mucosal immune responses of lymphotoxin-alpha deficient mice.[1]

1. Submitted for publication to *Clinical Immunology and Immunopathology,* manuscript number CLIN98-0078.

Chapter 1: Abstract

Lymphotoxin (LT-α) deficient (mutant) mice generate specific immune responses to orally administered immunogens despite having neither gut associated nor peripheral lymphoid tissues. The spleen, therefor, was expected to play a role in the generation of immune responses in these mutant mice. Mutant and wild-type (w.t.) mice were splenectomized and orally immunized with *S. typhimurium*. Splenectomy produced the most profound effects on serum and fecal IgA levels in mutant mice. Total and antigen specific serum and fecal IgA were increased in splenectomized w.t. mice but decreased in splenectomized mutant mice. Antigen specific serum IgG was decreased in both mutant and w.t. splenectomized mice while total IgG increased in splenectomized w.t. mice. Both splenectomized w.t. and mutant mice demonstrated a compensatory expansion of the lamina propria (LP) compartment characterized by a significant increase in the number of IgA spot forming cells (SFC). Mutant mice demonstrated further compensation for the loss of the spleen in the accelerated development of ectopic lymphoid tissues. We conclude that the spleen plays a prominent role as a lymphoid organ in mutant mice but its removal does not abolish immune responsiveness. Residual immune responsiveness in splenectomized mutant mice following oral immunization appears to be due to expansion and/or development of alternate effector compartments.

Chapter 2: Introduction

The generation of immune responses to antigens delivered across mucosal surfaces depends on events occurring in specific induction and effector tissues. Within the intestinal tract, cells comprising the Peyer's patches (PP) and mesenteric lymph nodes (MLN) provide the inductive arm while cells within the lamina propria (LP) provide the effector arm of immune responses generated against luminally delivered antigen (1-3). Luminal antigen is taken up by follicle associated epithelium and microfold (M) cells overlying the PP which may pass intact or processed antigen to underlying mucosal or PP professional antigen presenting cells (APC) or lymphocytes (4). PP nodules contain within them IgA secreting plasma cell precursors which, following appropriate costimulation, multiply and pass via lymphatic vessels to the MLN (5). Within the MLN, maturation occurs and many of these IgA B cells migrate back to the LP to serve effector functions (3).

Mice deficient in lymphotoxin-alpha (mutant) have neither grossly nor microscopically visible PP or MLN[1] and are thus expected to have impaired mucosal immune responses following oral antigen administration. The epithelial cells of the gastrointestinal tract, being functional in both antigen uptake and processing, may serve as APCs thereby replacing PP as inducers of specific immune responses (4, 5) in mutant mice. However, at least in wild-type (w.t.) mice, regulatory T cells, rather than helper T cells, may be induced by antigen presenting epithelial cells (6). The LP compartment of mutant mice appears to contain similar populations of cells compared to wt mice (see Part 2) but this compartment in such mice may be both an inductive and effector site.

The migratory paths and maturational processes which follow mucosal inductive events in mutant mice are unknown. Since mutant mice do not appear to have impaired lymphatic vessel formation (7, 8, and Part 3), a location which serves the function of the MLN may exist wherein lymphocytes from the gastrointestinal tract receive maturational signals prior to returning to the LP effector compartment. Recently it was shown that the blockage of lymphocyte traffic through lymph nodes resulted in increased trafficking of lymphocytes to the spleen (9). Interestingly, mutant mice have enlarged spleens, a phenotype which becomes more dramatic with age. Perhaps, in mutant mice, following mucosal inductive events, B lymphocytes travel in the expected pattern via lymphatic vessels to the vascular system and enter the spleen. In the spleen, the appropriate costimulation and maturational events may take place thus allowing mature isotype specific plasma cells to home back to the LP to carry out effector functions.

To determine if the spleen plays a dominant role in the generation of mucosal immune responses in mutant mice, splenectomized mutant and w.t. mice were dosed orally with *Salmonella typhimurium* and their immune responses were compared to those of non-splenectomized sham operated mice. Our results show that although splenic removal markedly affected the ability of mutant mice to generate mucosal IgA responses, such responses, both total and antigen specific, were not eliminated. In w.t. mice, in contrast, splenectomy resulted in elevated IgA responses, both systemically and mucosally. The LP provided the greatest degree of expansion in both splenectomized w.t. and mutant mice, however this appeared to contribute only to IgA responses. Splenectomy resulted in less profound changes in total serum IgG and IgM levels with

w.t. mice demonstrating increased and mutant mice demonstrating decreased responses. Significant expansion of the BM with respect to IgM, IgG, and IgA secreting cells was not observed indicating a minimal contribution towards the maintenance of antibody levels following oral immunization in splenectomized w.t. or mutant mice.

Finally, splenectomy led to the precocious development of ectopic lymphoid tissues in mutant mice. These lymph node-like foci were populated predominantly with IgM and IgG expressing $B220^{+}$ cells suggesting a possible role in the maintenance of serum Ig levels in splenectomized mutant mice. Our results further characterize the LT-α deficient phenotype and identify potential compensatory mechanisms for the generation of both systemic and mucosal immune responses in mice lacking major components of the immune system. We provide evidence that neither organized gut associated lymphoid tissues (GALT) nor the spleen are required for the generation of mucosal immune responses following oral antigen administration. Further, we show that in the absence of organized immune compartments, lymphocytes are capable of limited self-organization in alternate tissue sites.

Chapter 3: Materials and Methods

Mice

Development and identification of mice rendered genetically deficient in LT-α has been described previously (10). Original homozygous mutant female mice were bred to C57BL/6 males obtained from Jackson Laboratory (Bar Harbor, ME). All mutant mice used in the studies described here were the progeny of F4 heterozygous brother/sister interbreeding. C57BL/6 wild-type (w.t.) mice used were offspring of breeders obtained from the Jackson Laboratory. Mice were placed into experimental groups at 5-6 weeks of age. All mice were housed throughout the study in microisolator cages and maintained in a barrier facility at the Walters Life Sciences Building, University of Tennessee, Knoxville, and handled in accordance with institutional guidelines.

Surgical manipulation

Mice were anesthetized with a combination injectible mixture consisting of 1.1 mg xylazine, 3.3 ng butorphanol, and 0.01 ng ketamine in 200 μl sterile Ca^{2+}- and Mg^{2+}-free 1x physiologic buffered saline (PBS) delivered intramuscularly per mouse. Following sterile skin preparation, the spleen was located and exteriorized through a 1 cm left subcostal incision. In splenectomized mice, the splenic artery and vein were double ligated and the spleen removed. In sham operated mice, the spleen was replaced to the

abdominal cavity. The peritoneum and skin were closed in separate layers using sterile 5-0 absorbable suture. Mice were rested for 10 days prior to antigen administration.

Antigen and Administration

S. typhimurium strain BRD847, which expresses tetanus toxin fragment C (ToxC), was used under agreement with Medeva PLC (Surrey, UK) and has been described previously (11,12). Mice were held off feed and water for 3 hours, placed under general inhalant anesthesia (Metofane, Mallinckrodt Veterinary, Inc., Mundelein, IL), and dosed orally on Days 0, 10 and 20 with 100 µl (approximately 1.0×10^9 colony forming units (CFU)) of the bacterial cultures via an orogastric feeding needle. Bacterial cultures were serially diluted and plated on LB-ampicillin agar to confirm CFU given to mice.

Sample collection

Serum and fecal samples were collected on Days 0, 10, 20, and 28 days. Blood samples were obtained via capillary tube puncture of the retro-orbital venous sinus of Metofane anesthetized mice. Serum was separated following sample centrifugation and stored at -20° C until analysis. Individual 100 mg fecal samples were collected from mice, placed in 1 ml 0.1% sodium azide in 1x PBS, and vortexed for 15 minutes. Fecal slurries were then centrifuged for 8 minutes at 3000 x *g*, supernatant collected and stored at –20 °C until enzyme-linked immunosorbent assays (ELISA) could be performed.

Mice were terminated on Day 28 and bone marrow cells, LP lymphocytes (LPL), and whole spleen cells (sham mice) were collected. Briefly, spleens were aseptically removed and divided to allow for cell isolation and immunohistochemistry. For cell isolation, splenic portions were minced with scissors and gently pressed through a wire mesh screen into RPMI complete media (RPMI supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine). LPL were isolated following a digestion protocol described in detail elsewhere (13, and Part 2). Cells were then subjected to Percoll gradient centrifugation and LPL were recovered from the 40%-100% interface. BM cells were collected from femurs as described previously (14) and placed in cold complete RPMI.

All cell populations were suspended in cold complete RPMI. Red blood cells were lysed and cell populations adjusted to 1.0×10^7 cells/ml in complete RPMI media. Bone marrow (2 experiments) and LP (2 experiments) cell populations were pooled for each experimental group while splenocytes (3 experiments) were prepared individually for assays.

Determination of antibody responses by enzyme-linked immunosorbant assay (ELISA)

Total IgM, IgG and IgA and tetanus toxin fragment C (ToxC) specific IgG and IgA were determined from serum and fecal samples using a sandwich ELISA and following described protocols (15). Coating antibodies (goat anti-mouse IgG, Southern Biotechnology Assoc., Birmingham, AL; rabbit anti-mouse IgA, Zymed, South San Francisco, CA; goat anti-mouse IgM, Jackson Immuno Research, West Grove, PA; and

recombinant ToxC, Boehringer Mannheim, Indianapolis, IN) were diluted in carbonate buffer, pH 7.9. Standard antibodies of known concentrations (mouse IgG, Southern Biotech; purified mouse myeloma IgA, Zymed; and mouse myeloma IgM, Sigma) and samples were analyzed in duplicate and diluted in 1 x PBS as were secondary (HRP-goat anti-mouse IgG, Southern Biotech; biotin-goat anti-mouse IgA, Zymed; and HRP-rabbit anti-mouse IgM, Zymed) and tertiary (peroxidase-conjugated streptavidin, Jackson) antibodies. Samples and standards were incubated overnight at 4° C. Secondary and tertiary antibodies were incubated for 2 and 1 hours, respectively, at 37° C. Plates were developed using ABTS (2,2' –azino-di-(3-ethylbenzthiazoline) sulfonic acid, Sigma) solution (4.4 mg ABTS in 20 ml 0.1 M citric acid plus 20 ml 0.1 M dibasic sodium phosphate and 4 μl 30% H_2O_2). All samples were run in duplicate and average absorbance was read at 405 λ on an automated plate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA). Titers are expressed as a group mean, in ng/ml or mg/ml, with appropriate standard errors displayed.

Determination of immunoglobulin secreting cells by enzyme-linked immunosorbent spot (ELISPOT) assay

To quantitate the number of BM, spleen or LPL cells secreting IgA or IgG following antigen administration, nitrocellulose plates (Multiscreen-HA filtration plates, Millipore, Bedford, MA) were coated with either rabbit anti-mouse IgA at 2 μg/ml or goat anti-mouse IgG at 1 μg/ml following an established technique (16). 200 μl containing 2.0 x 10^6 cells were added and serially diluted 1:2 into sterile complete RPMI.

For detection of IgA secreting cells, biotinylated goat anti-mouse IgA diluted 1:2000 was applied for 2 hours at 37° C and followed by alkaline phosphatase conjugated streptavidine (Jackson) diluted 1:1000 for 1 hour at 37° C. For detection of IgG secreting cells, biotinylated rabbit anti-mouse IgG (Zymed) diluted 1:1000 and alkaline phosphatase conjugated streptavidin were applied as described above. Color was developed using BCIP/NBT substrate (5-bromo-4-chloro-3-indole-phosphate/nitro blue tetrazolium, (Sigma)) and spots counted on a dissecting microscope stage. Results are reported as mean spot forming cells (SFC) per million cells.

Histology

Following euthanasia, tissues were individually collected from mice and placed in periodate/lysine/paraformaldehyde (PLP) solution, stored at 4 °C for 24 hours, washed in 1x PBS, dehydrated, and infiltrated for embedding in low melting point (51-53 °C) paraffin as previously described (17). Serial 6 μm sections were cut on a microtome, placed on positively charged slides, and either stained with hematoxylin and eosin following standard techniques or stored at -20 °C until immunohistochemistry could be performed.

Immunohistochemistry

Slides with paraffin embedded tissue sections were deparaffinized, rehydrated, and prepared for specific cell staining as described (17). Primary antibodies (anti-mouse CD_4 (L3T4) or biotin-anti-mouse CD45R/B220, Pharmingen, San Diego, CA, and biotinylated

goat anti-mouse IgA or biotinylated rabbit anti-mouse IgG, Zymed) were incubated for 14-18 hours at 4 °C. Secondary antibody (biotinylated rabbit anti-rat polyclonal IgG, Pharmingen, for CD_4 only) was incubated for 4 hours at RT. Avidin-biotin horseradish peroxidase complex (ABC-HRP, Vector Labs, Burlingame, CA) was incubated for 30 minutes at RT. The substrate for ABC was Vector VIP (Vector) or DAB (3,3'-diaminobenzidine, Biogenex, San Ramon, CA). Tissue sections were counterstained with methyl green (Vector) and permanently cover slipped.

Statistics

The mean, standard error, and the significance of differences between groups using the two-tailed student's test were applied where indicated using 95% confidence limits. Data from sham operated and splenectomized mice are presented. There was no significant difference in any parameters measured between sham operated and intact mice, regardless of experimental group.

Chapter 4: Results

Splenectomy does not lead to increased mortality in mutant mice

Observations of LT-α mutant mice between the ages of 1 and 18 months reveal an increased splenic size which becomes more dramatic with age. To determine the role of the spleen in the immune responsiveness of mutant mice, the effects of splenectomy on systemic and mucosal immune responses was analyzed. The data presented here represent information from 55 mutant and 60 w.t. mice of which 23 mutant and 26 w.t. mice were splenectomized. As a group, splenectomized w.t. and mutant mice were clinically indistinguishable from intact or sham operated mice, even following oral administration of an attenuated strain of *S. typhimurium.* It should be noted, however, that all mice in this study were maintained in a barrier facility under sterile conditions and young eusplenic KO mice from this colony do suffer death losses when moved to conventional facilities.

Splenectomy results in decreased immune responsiveness in mutant mice

Following oral administration of recombinant *S. typhimurium* strain BRD847, eusplenic mutant mice generated measurable fecal and serum antibody responses to ToxC although levels were 3-8 fold lower than those generated in w.t. mice. Mutant mice have essentially 3 compartments in which effector lymphocytes can reside (spleen, BM, and LP) while w.t. mice possess these along with all peripheral lymph nodes. It was expected, therefor, that the spleen would play a more crucial role as a lymphoid organ in mutant versus w.t. mice. To test this idea, systemic and mucosal immune responsiveness to

orally administered *S. typhimurium* was compared in splenectomized and sham operated mutant and w.t. mice. The results indicate that splenectomy indeed was more consequential in mutant mice with the most profound effects on systemic and mucosal IgA production. Sham operated naïve mutant mice had mucosal IgA levels, as measured from fecal slurries, consistently and significantly lower than those levels measured in sham operated naïve w.t. mice. This effect was suspected to result from the lack of GALT in mutant mice. Immunization of sham operated w.t. and mutant mice led to proportional elevations of total fecal IgA levels such that total fecal IgA in w.t. mice remained over twice that of mutant mice. This correlation in responsiveness was lost, however, following splenectomy. The immune responsiveness in splenectomized mutant mice was greatly impaired as measured by total and antigen specific fecal IgA (**Figures 1a and b**, pages 144-145) as well as serum total IgA (data not shown). Conceivably, then, the spleen plays a major role as a lymphoid organ in mutant mice. Splenectomized w.t. mice, following immunization with BRD847, demonstrated increased total and antigen specific IgA in both serum (data not shown) and fecal slurries. Therefor, w.t. mice were better able to compensate for the loss of the spleen presumably due to the presence of peripheral lymphoid tissues and GALT.

A different scenario was observed in the effects of splenectomy on serum IgG levels. Splenectomy had little effect on total serum IgG levels in either w.t. or mutant mice in the naïve state. However, following immunization, splenectomized w.t. mice demonstrated increased total serum IgG levels while splenectomized mutant mice displayed a decrease in these levels (**Figure 2a**, page 146-147). The changes in levels of

total serum IgG, as well as total serum IgM (**Figure 3**, page 148), following splenectomy were not as dramatic as were the changes in total IgA levels. This may indicate that maintenance of serum IgG and IgM responses occur by compensation in the secondary lymphoid tissues. Analyses of antigen specific serum IgG production in splenectomized w.t. and mutant mice are recorded in **Figure 2b** (pages 146-147). Both w.t. and mutant splenectomized mice show decreased systemic responsiveness to mucosally administered *S. typhimurium* compared to sham operated controls. This decrease in IgG antigen specific responsiveness was more profound in mutant than w.t. splenectomized mice, but of less severity than the observed changes in IgA responsiveness.

Clearly, the mutant mouse is capable of compensating for the loss of the spleen to allow for the generation of systemic IgG and IgM and mucosal IgA responses to an orally administered antigen. However, these responses appear to be differentially compensated. Removal of the spleen prior to immunization did not completely abolish systemic and mucosal immune responsiveness of mutant mice to oral antigen. Compensatory mechanism were suspected in splenectomized mutant mice and tested for by evaluating changes in total IgG and IgA spot forming cells (SFC) in various immune compartments. Our findings, which are presented in **Figures 4a and b** (pages 149-150) and illustrated in **Figure 5** (page 151), show the greatest ability to compensate in both splenectomized w.t. and mutant mice is seen in the LP but only for IgA SFC. The number of IgA SFC in the BM did not significantly increase in either w.t. or mutant immunized mice following splenectomy (**Figure 4a**). Additionally, neither the BM nor LP appeared to significantly

compensate for IgG production following immunization of splenectomized mice (**Figure 4b**). The expansion of the LP compartment in splenectomized w.t. mice far exceeded that of mutant mice indicating a possible migration of IgA SFC from PP and MLN to the LP. Because PP and MLN are absent in mutant mice, expansion of existing LP effector cells is likely the result of local proliferation of IgA SFC. The lack of expansion of both the BM and LP to compensate for IgG SFC following splenectomy indicates the presence of further compensatory effector sites in both w.t. and mutant mice. These sites are presumed to be the existing lymph nodes in w.t. mice.

Mutant mice compensate for the loss of the spleen by the precocious development of ectopic lymphoid foci which may serve as sites of primary immune induction

Mutant mice, the related LT-β deficient mouse (10, 18), and mice lacking the tumor necrosis factor p55 receptor (19) are reported to develop ectopic lymph node-like structures. We have noted such structures in aged naïve mutant mice located primarily around vessels in high perfusion organs such as the liver and lung but also within the submucosal layer of intestinal segments. These foci could serve either or both of the following functions; repositories of lymphocytes unable to localize elsewhere due to the lack of secondary lymphoid tissues in mutant mice, or compensatory sites for Ig production. Mutant but not w.t. immunized mice developed such foci within 4 weeks post-splenectomy. Splenectomy resulted in the premature formation of such foci as none were noted in sham operated mutant or w.t. mice within this time period (**Figures 6a-d**, pages 152-156). In **Figure 7** (pages 157-161), hepatic (**a**) and pulmonary (**b**) foci show

large numbers of $B220^+$ cells. In **Figure 7c and d**, these B cells were shown to be predominantly IgG secreting. Staining for IgM also proved positive while that for IgA was present but minimal (data not shown). It is suspected that the ectopic lymphoid tissues observed in young (10-11 weeks) splenectomized mutant mice function as peripheral lymph nodes providing sufficient IgG and IgM production to compensate for the loss of the spleen.

Chapter 5: Discussion

This report deals with the issue of how the LT-α deficient mouse, which apart from a disorganized spleen, lacks morphologically evident secondary lymphoid organs and tissues such as Peyer's patches, can nonetheless generate immune responses following systemic (10, 20-24) and mucosal immunization. The data presented here indicate that the spleen is largely responsible for IgG and IgM responses, but splenic removal in mutant mice has more consequence on, but did not totally abrogate, mucosal IgA responses. By way of contrast, splenectomy of w.t. mice actually resulted in elevated mucosal as well as systemic IgA responses. Residual responses were assumed to derive from the lamina propria since the number of IgA producing cells was elevated in this compartment following splenectomy. As reported previously, mutant mice may develop ectopic accumulations of lymphoid tissue as they age. Curiously, splenectomy of mutant mice led to the precocious development of such ectopic lymphoid foci and these are assumed to act as additional compensatory tissues, at least for serum IgM and IgG production. Our results serve to demonstrate that the immune system shows great plasticity and that other lymphoid tissues take on more prominent roles in immune induction in the absence of normal lymphoid organs.

Based on studies in humans, the murine spleen may receive 20-30% of celiac artery blood flow (25) and contains 20-30% of total blood volume (26). While the splenic lymphocyte population is dynamic, splenectomy in normal animals results in variable impairment in immune responsiveness (27) presumably due to abundant secondary lymphoid tissues. LT-α deficient mice lack all secondary lymphoid tissues

thus splenectomy was expected to profoundly impact not only their immune responsiveness but also their ability to survive infections. Unexpectedly, splenectomized mutant mice retained the ability, although reduced, to generate systemic and mucosal immune responses to an attenuated Salmonella strain and suffered neither increased morbidity nor mortality. However, had these mice been maintained in a dirty environment or had the study period been expanded, outcomes may have shown greater variation.

Splenectomy did affect immune responsiveness following enteric immunization but the outcome was different in w.t. and mutant mice. Mutant mice demonstrated the most profound effects at the level of IgA production while compensation for the maintenance of pre-surgical IgA levels appeared to function only in splenectomized w.t. mice. The induction of IgA responses most often occurs at mucosal surfaces following appropriate stimulation of resident GALT B lymphocytes (3). Because mutant mice lack GALT, their impaired mucosal responses to orally administered BRD847 were expected. The role played by the spleen in generating either IgA responses or the IgA committed B cells which populate the GALT following oral antigen administration is unknown. However, in mutant mice, this organ may conceivably be involved in isotype switching and cell population expansion (27). These roles are presumably played by secondary lymphoid tissues in w.t. mice.

Splenectomized mutant and w.t. mice appeared to utilize the LP compartment as an alternate effector site for the production of IgA as evidenced by the increase in numbers of LP IgA SFC. Splenectomized mutant mice showed further expansion of the

LP compartment post-immunization indicating the LP may compensate, at least partially, for mucosal immune responses. The expansion of the LP was greatest in w.t. mice immunized post-splenectomy. The organization and resident lymphocyte populations of GALT in w.t. mice may have allowed for cellular expansion and increased IgA production following oral immunization. This plasticity was lacking in mutant mice apparently due to the lack of organized GALT. Perhaps the role of the spleen in mutant mice includes organization and co-localization of cellular components to allow for amplification of immune responses.

Total serum IgG and IgM levels remained normal in splenectomized w.t. and mutant mice with alterations seen only after immunization. The ability of splenectomized mutant mice to maintain these serum Ig levels were suspected to be due to compensatory input from lymphocyte containing tissues such as the LP or BM. However, there was little evidence of increased involvement of the LP and BM in the production of serum IgG following splenectomy but there was more rapid development of ectopic lymphoid foci which expressed IgG (as well as and IgM) in mutant mice. Alimzhanov, et. al., (18) observed lymphocytic infiltrations into parenchymal organs of 8 week old eusplenic KO mice housed in a conventional facility. These lymphocytes were mainly B ($B220^+$) and $CD4^+$ T cells. Additionally, Banks, et.al. (10), observed similar cellular accumulations in older conventionally reared eusplenic mutant mice. We observed these infiltrations only in splenectomized mutant mice at 10-11 weeks of age and not in splenectomized w.t. or in any sham operated mice. However, they may have

developed in these other groups had our observation period been longer or had our mice been house in a more antigenically challenging environment.

Clearly, in the absence of secondary lymphoid tissues, specific lymphocyte populations are recruited to parenchymal organs. These foci, being microscopic, were too small to remove for functional evaluation to formally prove their contribution to serum Ig levels. However, the maintenance of serum IgM and IgG levels by mutant mice may be due, in part, to Ig production at these ectopic lymph node like structures. Sayles, et. al. (28) noted similar cellular accumulations within the livers of splenectomized mice following intravenous plasmodium infection. This group suggested that in the absence of a spleen, other tissues, including the liver, may develop into sites of antigen processing and presentation. Given that the spleen is the only peripheral lymphoid organ capable of serving this function in the mutant mouse, removal of the spleen would necessitate a detour of certain cell populations. Currently, the mechanisms and stimuli for cell entry to alternate tissue sites are unknown but are likely due to more than just lymphocyte homelessness. Since the activation state of lymphocytes determines their specific tracking patterns, a difference in both lymphocyte surface adhesion molecule and vascular addressin expression in mutant mice may explain these ectopic foci (Part 3). It has already been shown that differences in the intra-splenic expression of both peripheral node addressin (PNAd) and mucosal addressin (MadCam-1) exist in KO mice (8, 28). An alternate explanation for the development of ectopic lymphoid foci comes from the recent report of impaired elimination of reactive oxygen species in mice lacking the p55 tumor necrosis factor receptor (TNFR1) (19). The diminished production of manganese

superoxide dismutase (MnSOD) results in increased tissue damage following parasitic infection characterized by the infiltration of mononuclear cells and presumably due to unchecked production of reactive oxygen species by local TNFR1 deficient macrophages. While the mice used in these studies were maintained under near sterile conditions and had no evidence of bacteremia, it is possible that the lack of homotrimeric LT-α signaling at the TNFR1 plays a role in the lymphocytic tissue infiltration seen in LT-α deficient mice.

While we have not determined the exact inductive and effector sites necessary for the generation of immune responses following oral antigen administration in mutant mice, our data identify the spleen as important but non-essential and the LP as an alternate immune effector site. Splenectomized mutant mice are impaired but capable of mounting measurable mucosal and systemic immune responses to an orally administered T-dependent antigen. They are further capable of forming ectopic lymphoid-like structures which have a high percentage of IgG secreting cells and may, in fact, serve as sites of antigen processing and presentation. Splenectomized mutant mice obviously develop alternate strategies to allow humoral responses but at greatly impaired levels. The recent report of small intestinal cryptopatches which appear to be the origin of intraepithelial lymphocytes (29) validates the potential existence of compensatory lymphoid tissues. We conclude, then, based on the findings presented here, that the spleen, PP, and MLN are not absolutely required for the development of gut mucosal immune responses. It is doubtful, however, and highly unlikely, that animals rendered similarly deficient would survive outside the confines of a nearly sterile environment.

Nonetheless, the LT-α deficient mouse model provides a means of dissecting the components of the immune system.

Figure 1: Comparison of total and ToxC specific fecal IgA responses following splenectomy. Splenectomy results in profound impairment of mucosal immune responses in LT-α deficient but not w.t. mice following oral administration of *S. typhimurium* strain BRD847. Graphs serve to compare total (a) and antigen (ToxC) specific fecal IgA responses (b) between intact (sham) and splenectomized (surg) wild-type (w.t.) and LT-α deficient (mutant) mice. Days of sampling are indicated by colored bars. Differences between w.t. and mutant mice as well as sham operated and splenectomized mice are significant ($p < 0.005$).

Splenectomy impairs mucosal IgA responses more profoundly in mutant than w.t. mice

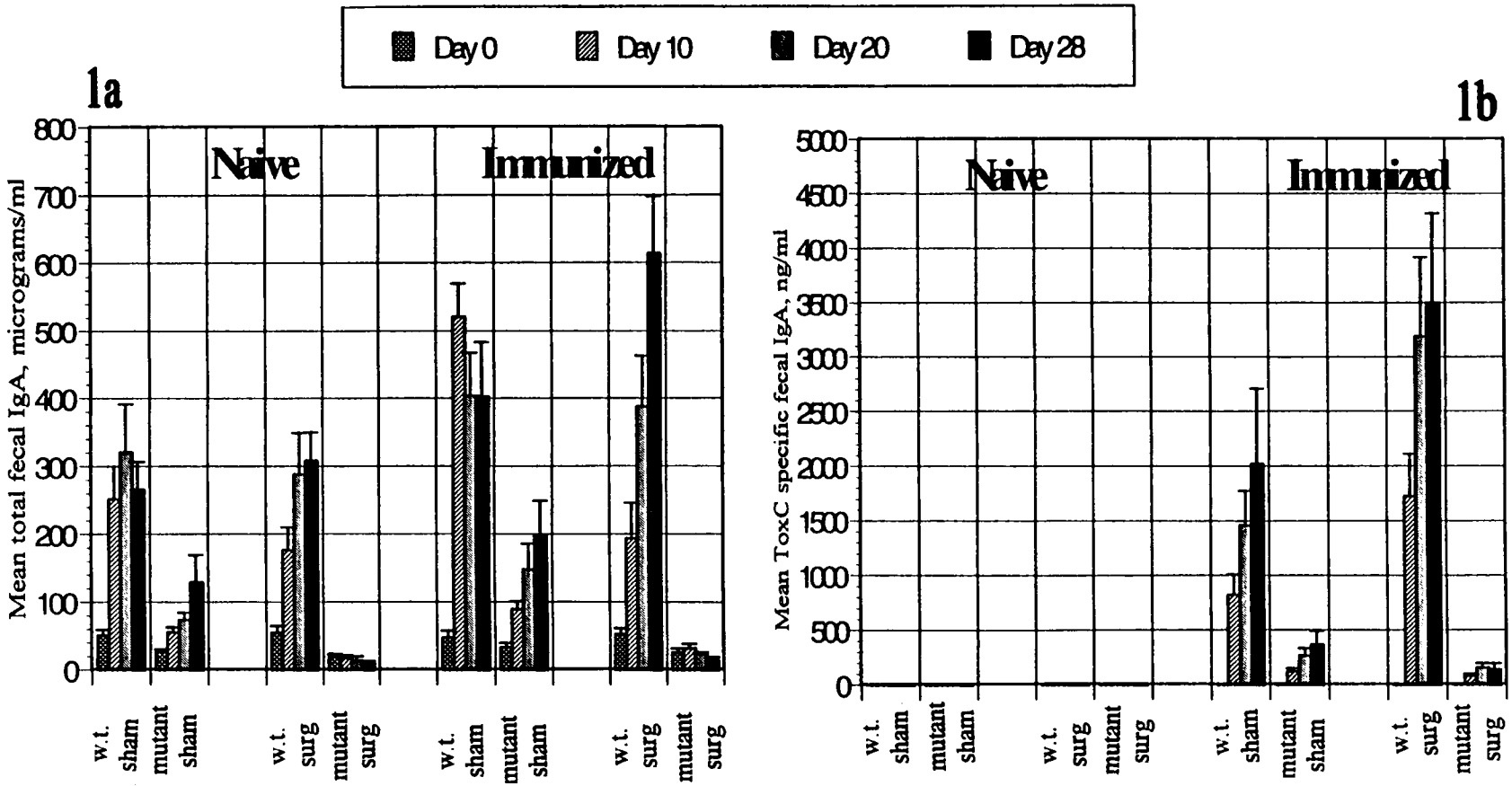


Figure 2: Comparison of total and ToxC specific serum IgG responses following splenectomy. Splenectomy results in decreased antigen specific serum IgG in both w.t. and mutant mice (b) but this correlation does not hold true for total serum IgG responses (a). W.t. splenectomized (w.t. surg) mice exhibit elevations in total serum IgG while mutant splenectomized (mutant surg) mice show a reduction in total serum IgG following oral antigen administration and over time. Sham operated eusplenic (sham) mice and splenectomized naïve mice demonstrated little variation in total serum IgG levels. Differences between w.t. and mutant mice as well as sham operated and splenectomized mice are significant ($p < 0.05$).

Splenectomy has similar effects on ToxC specific serum IgG but opposite effects on total IgG in KO mice as compared to wt mice

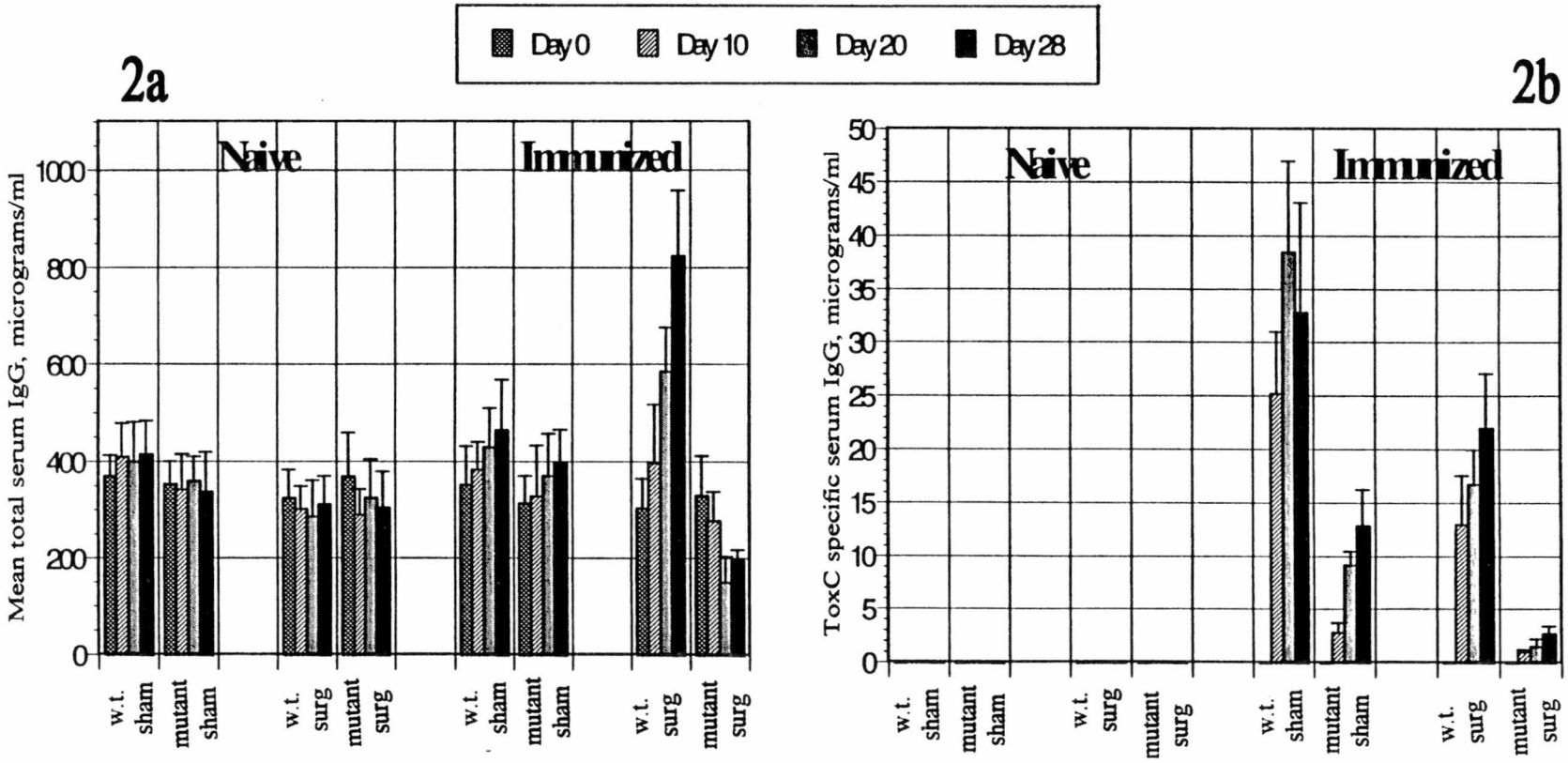


Figure 3: Total and antigen specific serum IgM levels

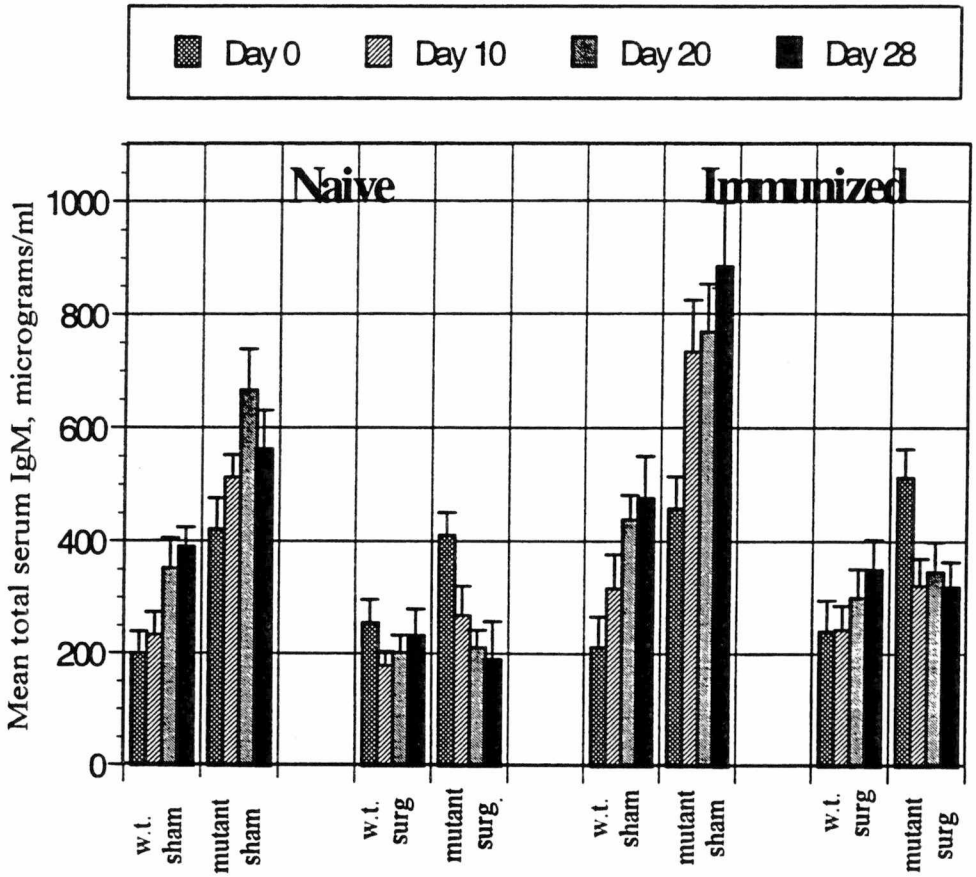
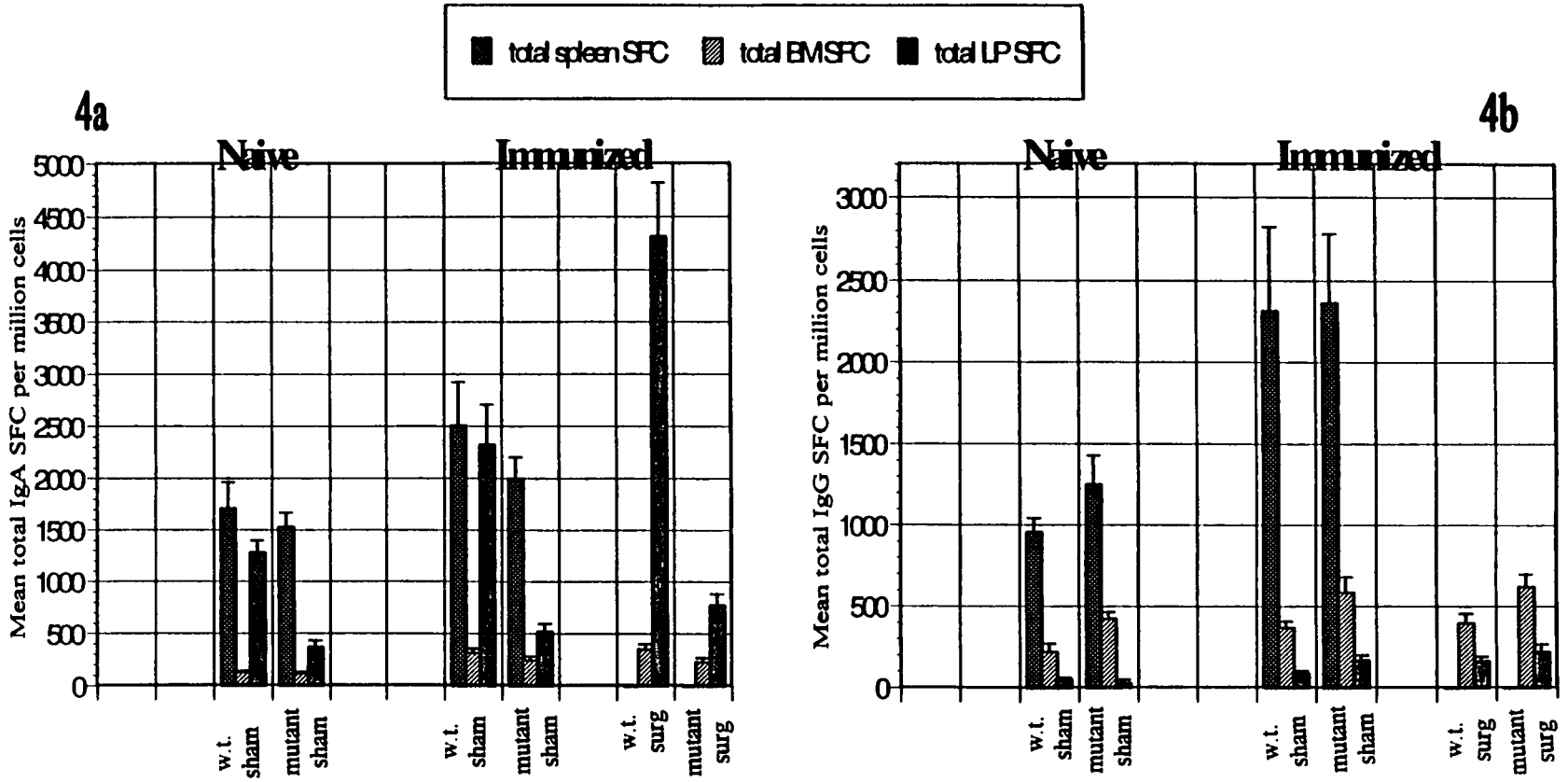


Figure 3: Splenectomy caused a reduction in total serum IgM levels in both KO and wt mice. KO mice have higher resting serum IgM levels than wt mice do. This difference is maintained following immunization. Splenectomy has a more profound effect on IgM levels in KO than wt mice ($P < .0005$ in KO splenectomized mice versus KO sham operated mice following immunization). All values are expressed as μg/ml. Sham = sham operated, Surg = splenectomized.

Figure 4a and 4b: Location of suspected immune compensatory compartments in mutant and w.t. mice. Only the lamina propria (LP) compartment showed appreciable expansion following splenectomy (surg) but this was true for only mucosal IgA production (4a). W.t. and mutant sham operated eusplenic (sham) mice had numbers of IgA and IgG (4b) spot forming cells (SFC) from spleen and bone marrow (BM) with significant differences ($p < .05$) seen only in the LP compartment of mutant mice.

Location of suspected compensatory compartments in splenectomized mice



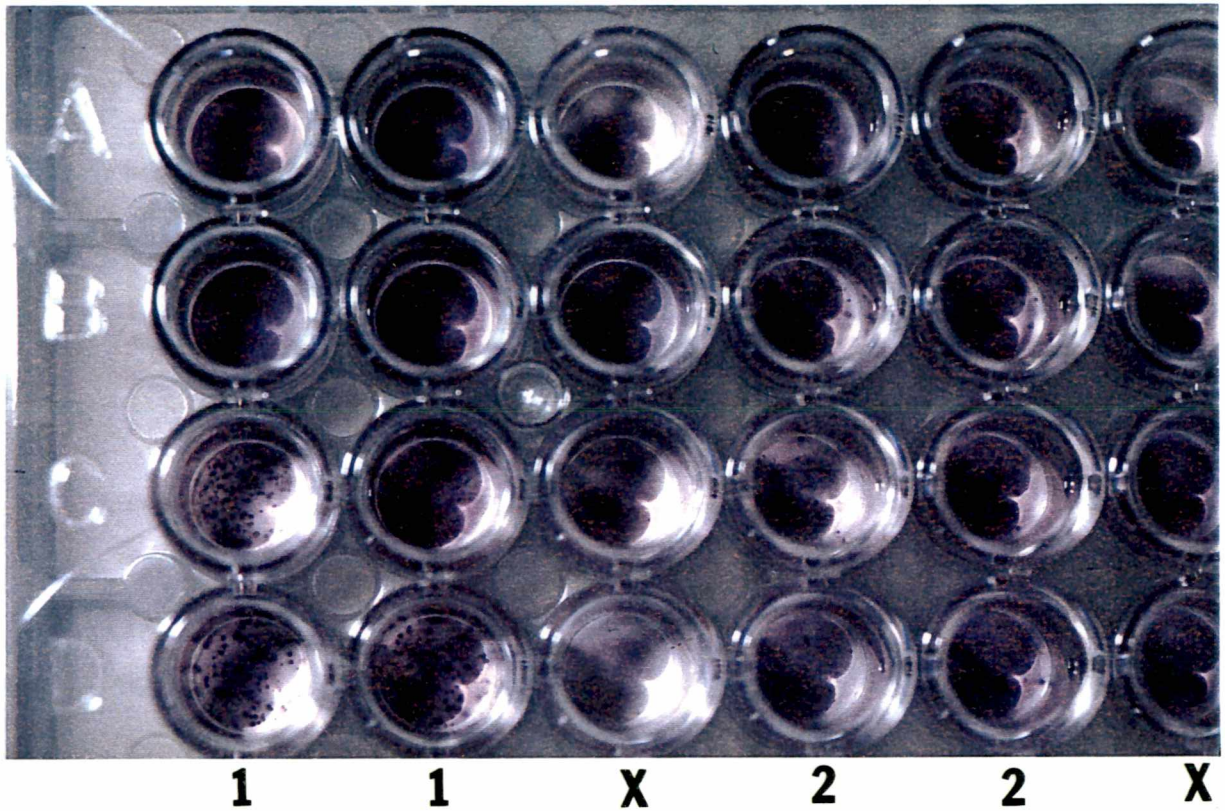
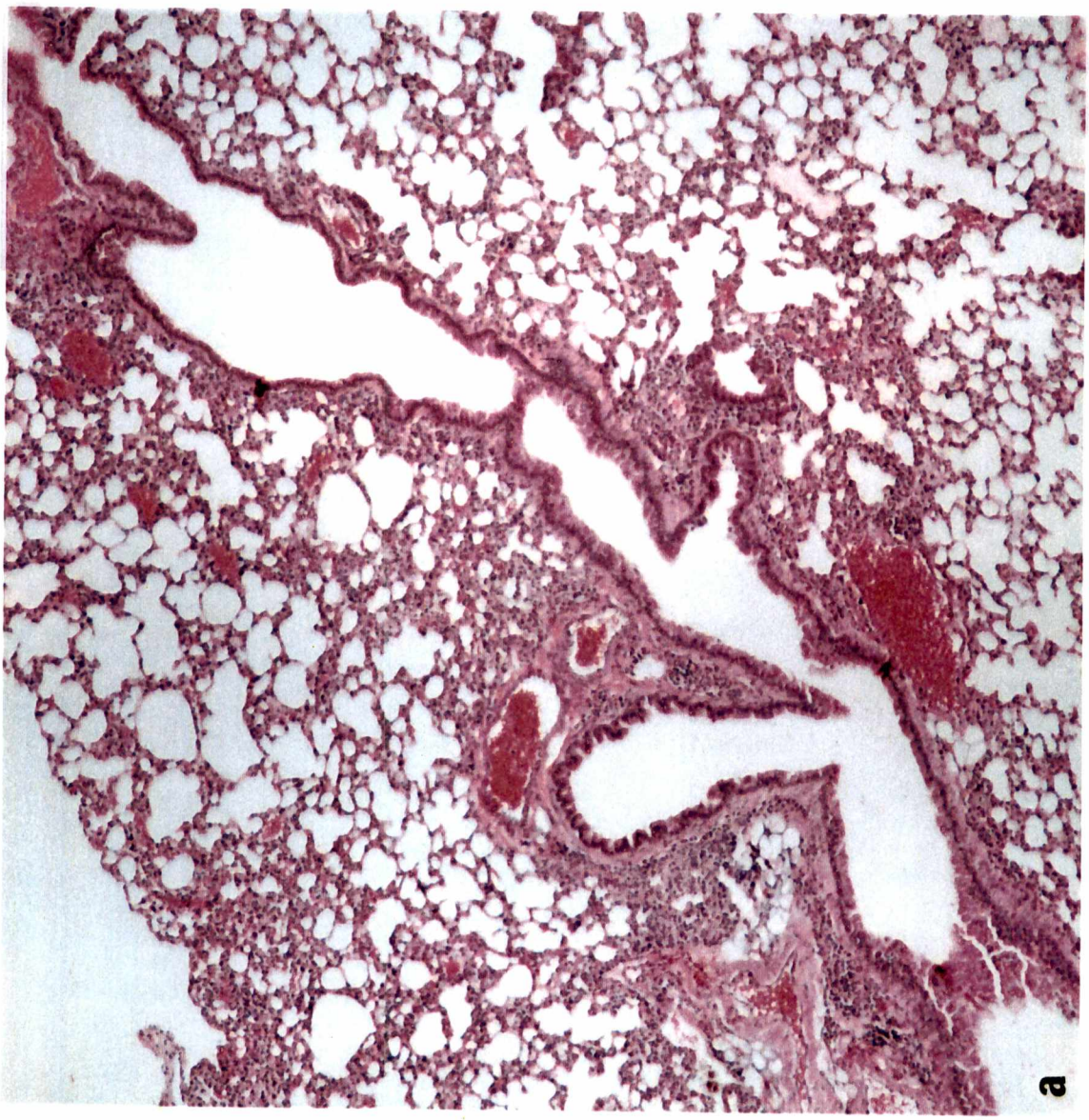
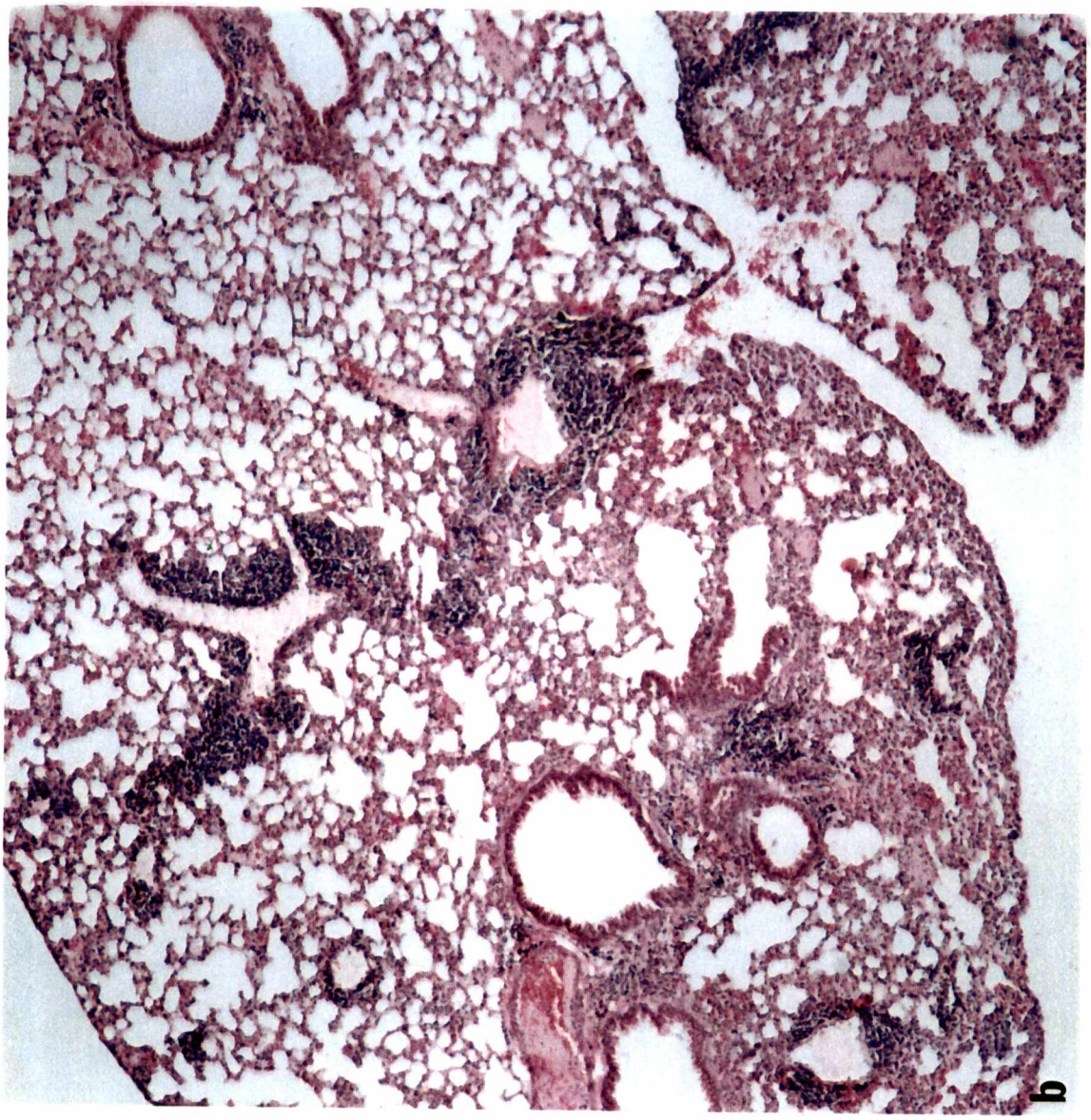
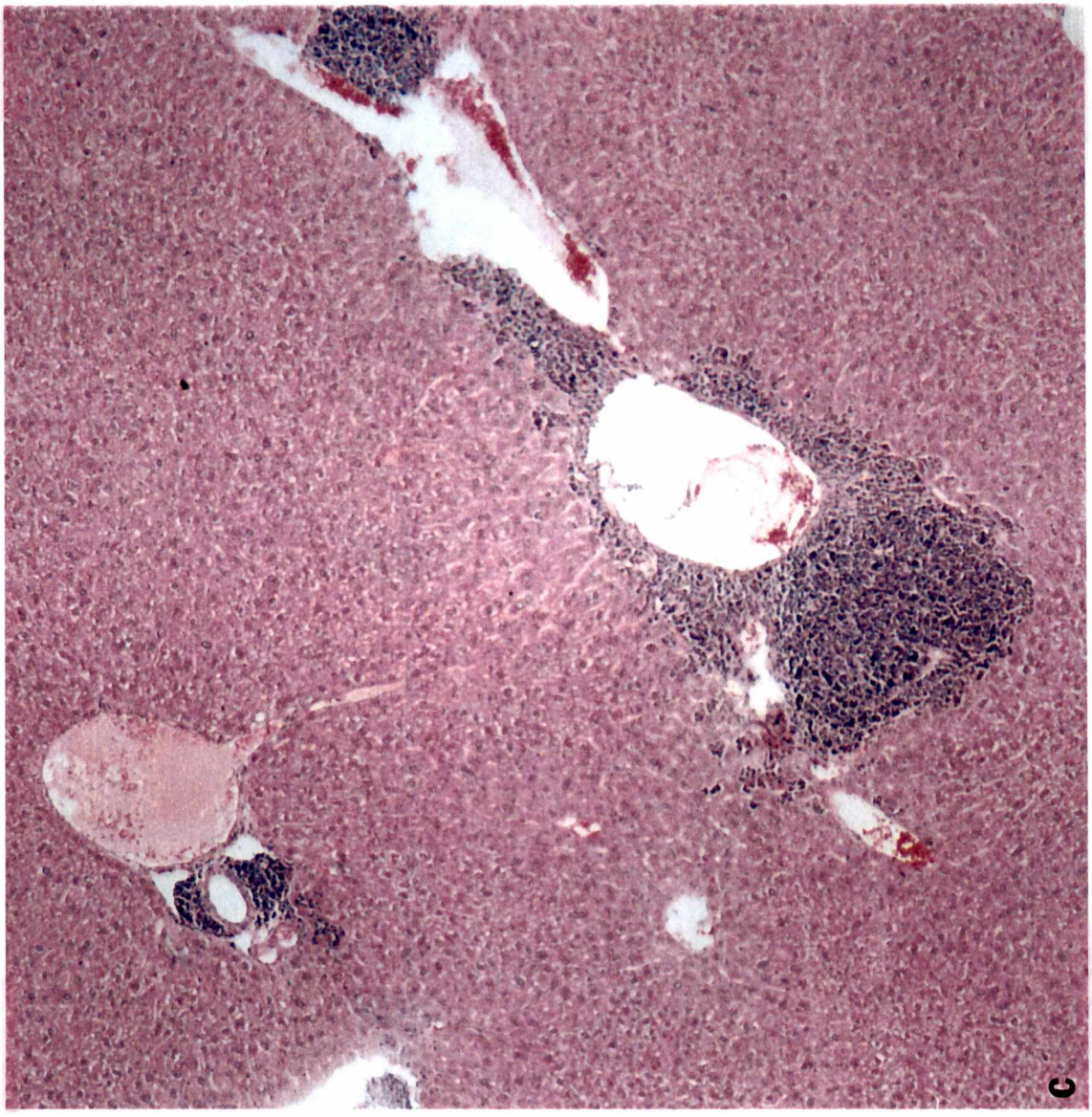


Figure 5: Representative ELISPOT results demonstrating differences in LP IgA spot forming cells between w.t. splenectomized (1) and mutant splenectomized (2) mice. Columns labeled X contained only media. Counts would be made from row D and averaged and would represent the number of SFC/1.25 x 10^6 cells.

Figures 6a-d: Splenectomy results in the precocious development of ectopic lymphoid foci in mutant but not w.t. mice. All photos are hematoxylin and eosin stained 6 μm paraffin embedded tissue sections collected on Day 28 of experimentation and magnified 25X (a and b) or 50X (c and d). Dark blue-purple nuclei represent infiltrating mononuclear cells. Section "a" is from a w.t. splenectomized mouse. Sections "b-d" are from a mutant splenectomized mouse and are lung, liver, and cecum, respectively.


B


b


c

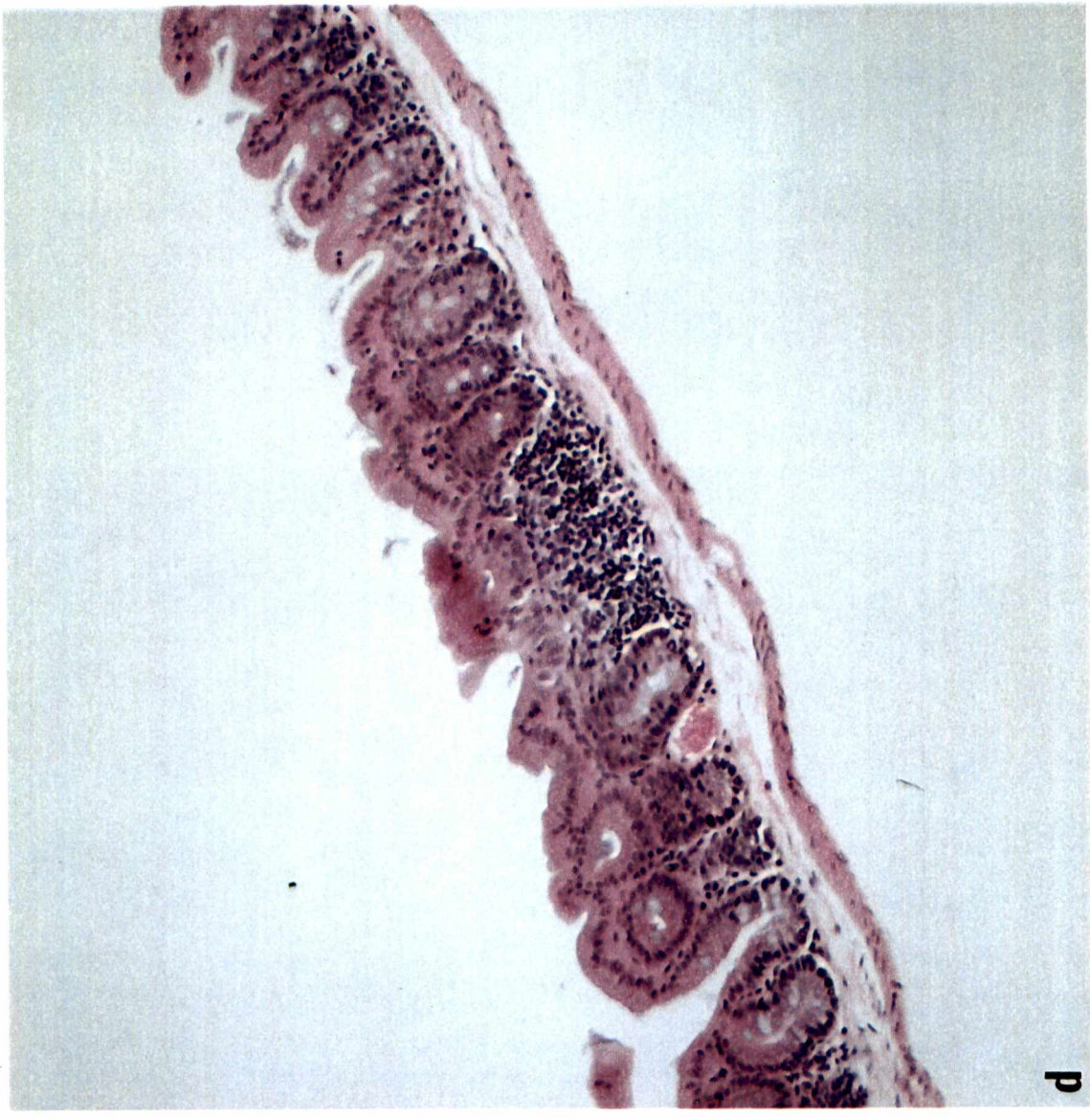
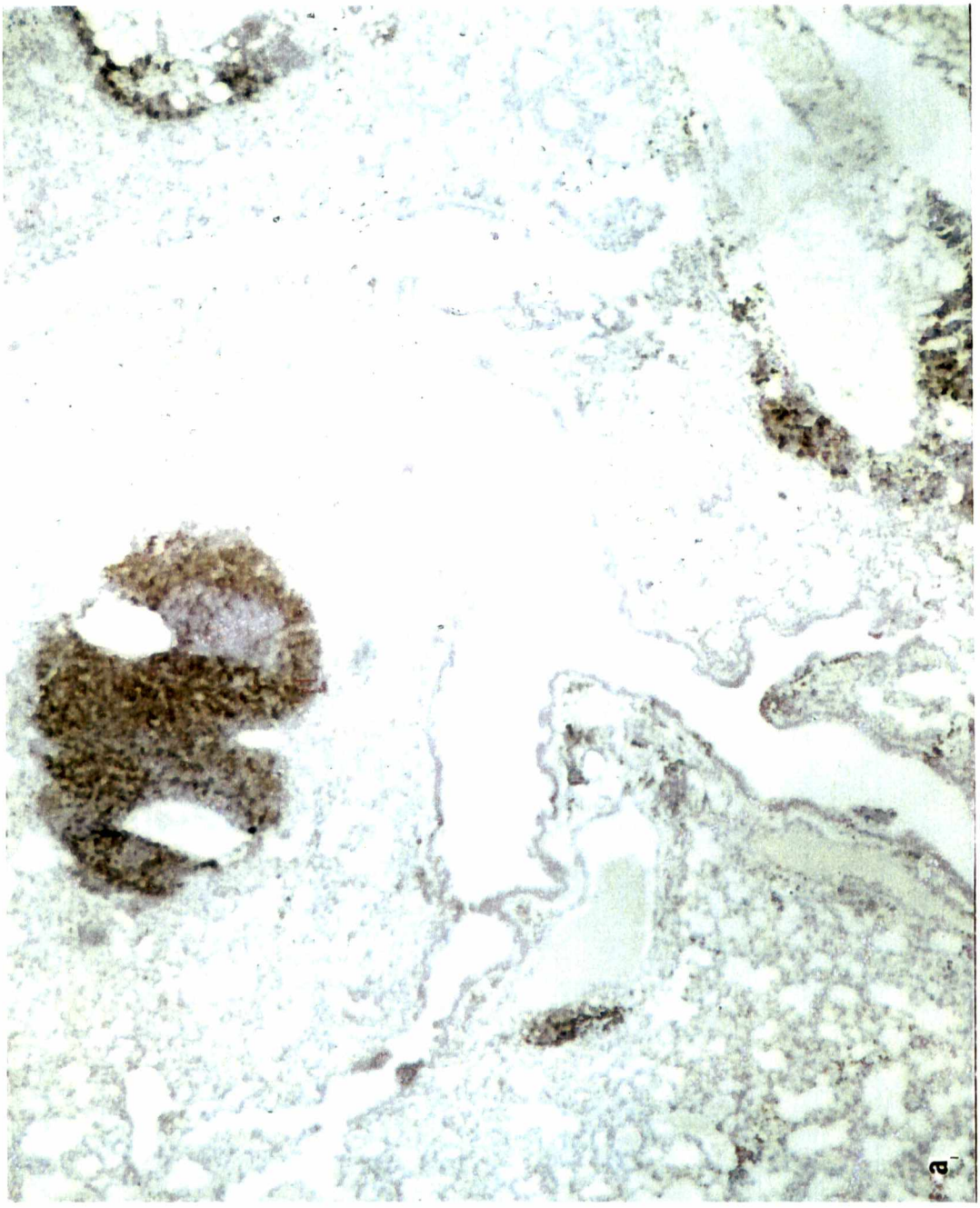
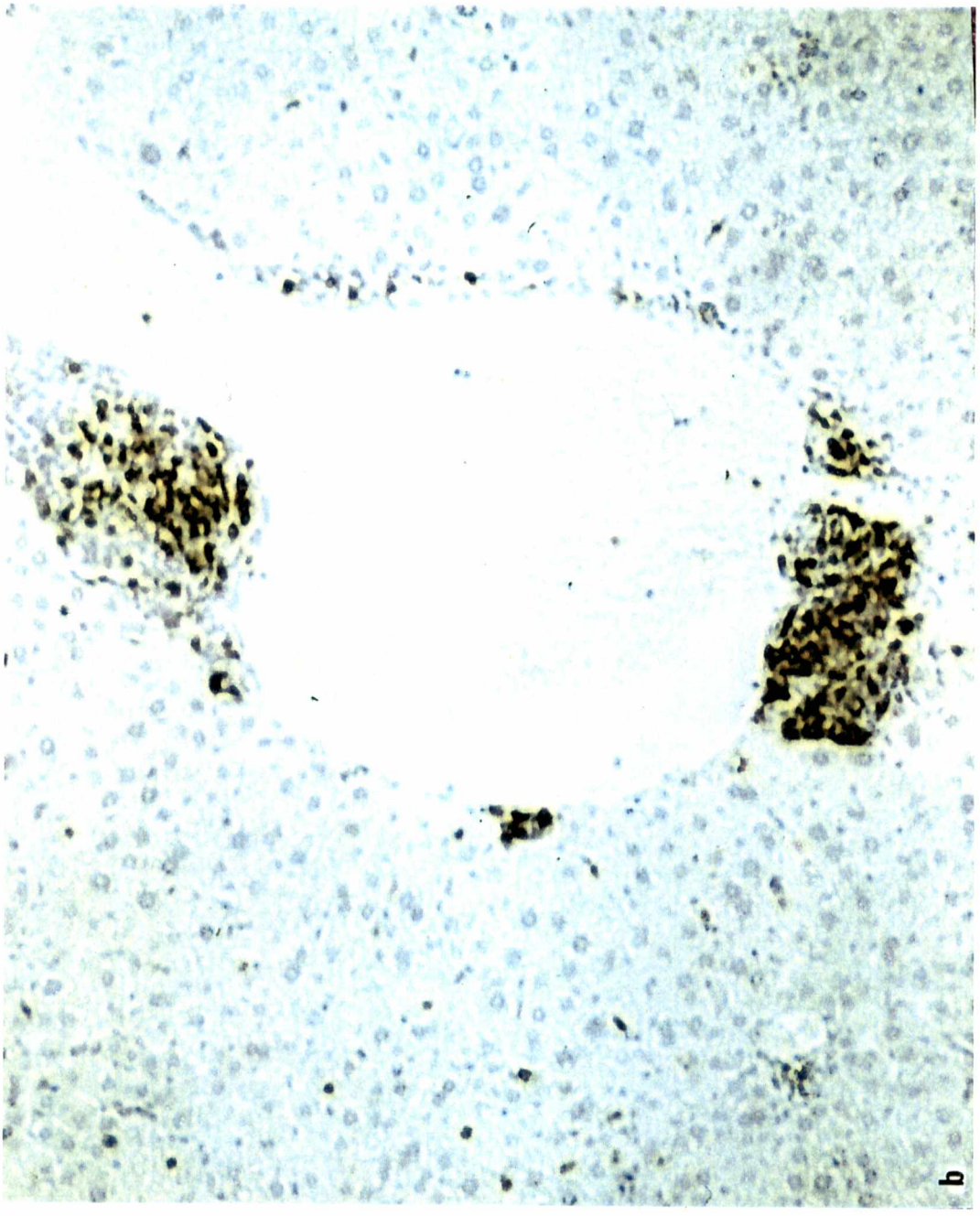
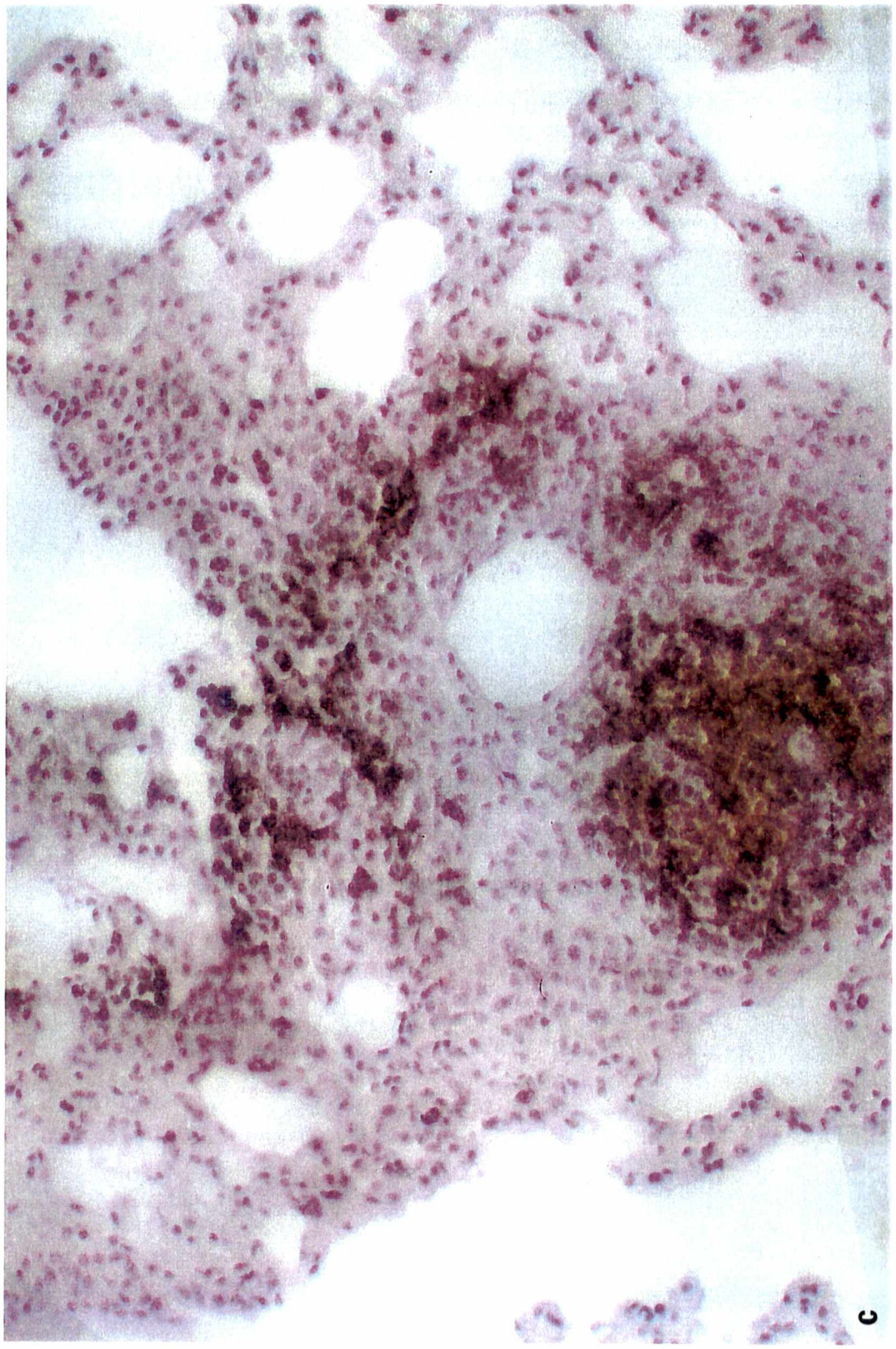
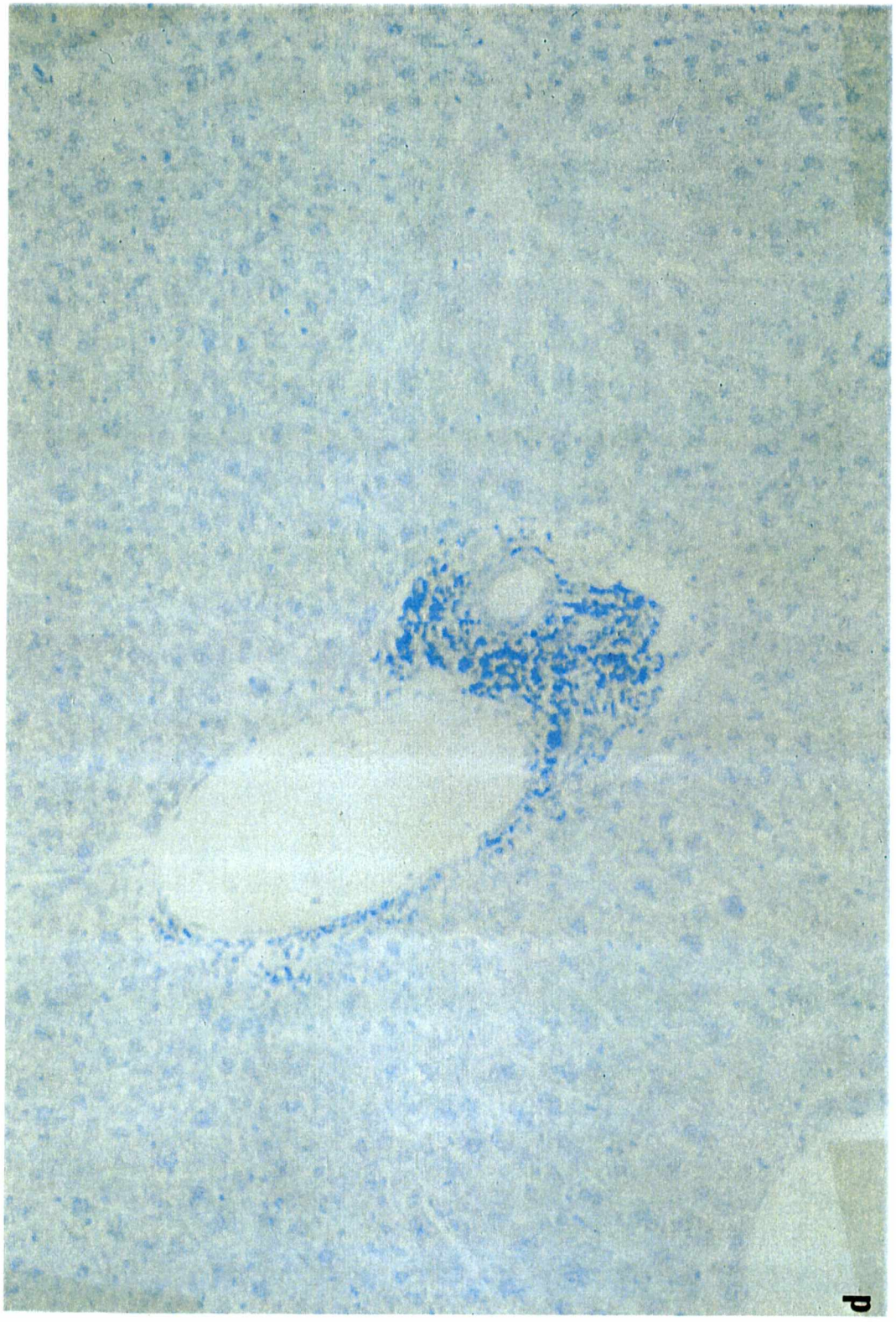

P

Figure 7a-d: The ectopic lymphoid foci which form in splenectomized mutant mice stain positively for B220 (a and b), indicating they are b cells, and for IgG (c and d), indicating surface expression and perhaps active secretion of this antibody (see text for details of staining). Sections "a" and "c" are paraffin embedded lung sections and sections "b" and "d" are similarly prepared liver sections. Magnification is 50X for "a" and "b" and 100X for "c" and "d".


a


b


c


D

Literature Cited

1. McDermott, M. R., Horsewood, P., Clark, D. A., and Bienenstock, J. 1986. T lymphocytes in the intestinal epithelium and lamina propria of mice. *Immunology* 57: 213-218.

2. Beagley, K. A., Eldridge, J. H., Lee, F., Kiyono, H., Everson, M. P., Koopman, W. J., Hirano, T., Kishimoto, T., and McGhee, J. R. 1989. Interleukins and IgA synthesis: Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. *J. Exp. Med.* 169: 2133-2148.

3. Mowat, A. M. and Viney, J. L. 1997. The anatomical basis of intestinal immunity. *Immunol. Rev.* 156: 145-166.

4. Kato, T. and Owen, R. L. 1994. Structure and function of intestinal mucosal epithelium. *In:* Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGhee, J. R., and Bienenstock, J., Eds., Handbook of Mucosal Immunology, Academic Press Inc, San Diego, CA, pp. 11-26.

5. Phillips-Quagliata, J. M. and Lamm, M. E. 1994. Lymphocyte homing to mucosal effector sites. *In:* Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGhee, J. R., and Bienenstock, J., Eds., Handbook of Mucosal Immunology, Academic Press Inc, San Diego, CA, pp. 225-239.

6. Mayer, L. and Shlien, R. 1987. Evidence for function of Ia molecules on gut epithelial cells in man. *J. Exp. Med.* 166: 1471-1483.

7. Rennet, P. D., Browning, J. L., Mebius, R., Mackay, F., and Hochman, P. S. 1996. Surface lymphotoxin α/β complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184: 1999-2006.

8. Sacca, R., Turley, S., Soong, L., Mellman, I., and Ruddle, N. H. 1997. Transgenic expression of lymphotoxin restores lymph nodes to lymphotoxin-α-deficient mice. *J. Immunol.* 159: 4252-4260.

9. Tripp, R. A., Topham, D. J., Watson, S. R., and Doherty, P. C., Bone marrow can function as a lymphoid organ during a primary immune response under conditions of disrupted lymphocyte trafficking. *J. Immunol.* 158, 3716-3720, 1997.

10. Banks, T. A., Rouse, B. T., Kerley, M. T., Blair, P. J., Godfrey, V. L., Kuklin, N. K., Bouley, D. M., Thomas, J., Kanangat, S., and Mucenski, M. L., Lymphotoxin-α-deficient mice: Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155, 1685-1693, 1995.

11. Chatfield, S. N., Charles, I. G., Makoff, A. J., Oxer, M. D., Dougan, G., Pickard, D., Slater, D., and Fairweather, N. F.,. Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. *Bio/Technology.* 10, 888-892, 1992.

12. Karem, K. L., Chatfield, S., Kuklin, N., and Rouse, B. T., Differential induction of carrier antigen-specific immunity by *Salmonella typhimurium* live-vaccine strains after single mucosal or intravenous immunizations of BALB/c mice. *Inf. Immun.* 63(12), 4557-4563, 1995.

13. Lefrancois, L. and Lycke, N., Isolation of mouse small intestinal intraepithelial lymphocytes, Peyer's patch, and lamina propria cells. *In* "Current Protocols in Immunology" (J.E. Coligan, A.M. Kruisbeck, D.M. Margulies, E.M. Shevach, and W. Strober, Eds.), pp. 3.19.1-3.19.16, John Wiley and Sons, Inc., USA, 1996.

14. Falk, L. A., Macrophages and monocytes. *In* "Current Protocols in Immunology" (J.E. Coligan, A. M. Kruisbeck, D. M. Margulies, E. M. Shevach, and W. Strober, Eds.), pp. 14.0.3-14.1.9, John Wiley and Sons, Inc., USA, 1995.

15. Hornbeck, P., Isotype determination of antibodies. *In* "Current Protocols in Immunology" (J. E. Coligan, A. M. Kruisbeck, D. M. Margulies, E. M. Shevach, and W. Strober, Eds.), pp. 2.2.1-2.2.2, John Wiley and Sons, Inc., USA, 1991.

16. Lycke, N. Y., Measurement of immunoglobulin synthesis using the ELISPOT assay. *In* "Current Protocols in Immunology" (J. E. Coligan, A. M. Kruisbeck, D. M. Margulies, E. M. Shevach, and W. Strober, Eds.), pp. 7.14.1-7.14.9, John Wiley and Sons, Inc., USA, 1996.

17. Whiteland, J. L., Nicholls, S. M., Shimeld, C., Easty, D. L., Williams, N. A., and Hill, T. J., Immunohistochemical detection of T-cell subsets and other leukocytes in paraffin-embedded rat and mouse tissues with monoclonal antibodies. *J. Histochem. Cytochem.* 43(1), 313-320, 1995.

18. Alimzhanov, M. B., Kuprash, D. V., Kosco-Vilbois, M. H., Luz, A., Turetskaya, R. L., Tarakhovsky, A., Rajewsky, K., Nedospasov, S. A., and Pfeffer, K., Abnormal development of secondary lymphoid tissues in lymphotoxin β–deficient mice. *Proc. Natl. Acad. Sci. USA.* 94, 9302-9307, 1997.

19. Castinos-Velez, E., Maerlan, S., Mosorio, L., Aberg, F., Biberfeld, P, Orn, A., and Rottenberg, M. C. 1998. *Trypanosoma cruzi* infection in tumor necrosis factor receptor p55-deficient mice. *Infect. Immun.* 66(6): 2960-2968.

20. De Togni, P., Goellner, T., Ruddle, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. C., Carlson, R., Shornick, L. P., Strauss-Schoenberger, J., Russell, J. H., Karr, R., and Chaplin, D. D. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264: 703-707.

21. Matsumoto, M., Fu, Y.-X., Molina, H., Huang, G., Kim, J., Thomas, D. A., Nahm, M. H., and Chaplin, D. D. 1997. Distinct roles of lymphotoxin α and the type I tumor necrosis factor (TNF) receptor in the establishment of follicular dendritic cells from non-bone marrow-derived cells. *J. Exp. Med.* 186(12): 1997-2004.

22. Mackay, F., Majeau, G. R., Lawton, P., Hochman, P. S., and Browning, J. L. 1997. Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice. *Eur. J. Immunol.* 27: 2033-2042.

23. Fu, Y.-X., Huang, G., Matsumoto, M., Molina, H., and Chaplin, D. D. 1997. Independent signals regulate development of primary and secondary follicle structure in spleen and mesenteric lymph node. *Proc. Natl. Acad. Sci. USA.* 94: 5739-5743.

24. Fu, Y.-X., Huang, G., Wang, Y., and Chaplin, D. D. 1998. B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin α-dependent fashion. *J. Exp. Med.* 187(7): 1009-1018.

25. Ganong, W. F. 1993. Circulation through special regions. *In:* Dolan, J. and Langans, C., Eds., Review of Medical Physiology, Appleton and Lange, East Norwalk, CT, pp. 551-570.

26. Jain, N. C. 1986. Blood volume and water balance. *In: Schalm's* Veterinary Hematology, pp. 87-102, Lea and Febiger, Philadelphia, PA, pp. 87-102.

27. Sayles, P. C., Yanez, D. M., and Wassom, D. L. 1993. *Plasmodium yoelii:* Splenectomy alters the antibody responses of infected mice. *Exp. Parasit.* 76: 377-384.

28. Gonzales, M., Mackay, F., Browning, J. L., Kosco-Vilbois, M. H., and Noelle, R. J. 1998. The sequential role of lymphotoxin and B cells in the development of splenic follicles. *J. Exp. Med.* 187(7): 997-1007.

29. Saito, H., Kanamori, Y., Takemori, T., Nariuchi, H., Kubota, E., Takahashi-Iwanaga, H., Iwanaga, T., and Ishikawa, H. 1998. Generation of intestinal T cells from progenitors residing in gut cryptopatches. *Science* 280: 275-278.

Part 6: Demonstration of impaired HSV-1 specific cytotoxic T lymphocyte function in lymphotoxin-alpha deficient mice

Chapter 1: Abstract

Lymphotoxin-alpha (LT-α) deficient mice demonstrate impaired humoral immune responsiveness following systemic and oral administration of several different antigens. This impairment is thought to directly result from impaired signaling through receptors at which LT-α interacts; the LT-β receptor which results in the failure of lymph node formation and lymphoid tissue germinal center development; and the p55 tumor necrosis factor alpha receptor (p55TNFR) which results in failure of Peyer's patch development. The signaling of homotrimeric LT-α through the p55TNFR may induce cytotoxic activity since signaling at this receptor induces macrophage activation and is involved in the clearance of several viral and intracellular bacterial infections. The activation of other cytotoxic functions such as carried out by cytotoxic T cells (CTL) and natural killer cells (NK) can be achieved by triggering the LT-β receptor by heterotrimeric LT-α_1:LT-β_2. We hypothesized that LT-α deficient mice would demonstrate deficient CTL and/or NK cell killing due to the inability of these mice to trigger either the p55TNFR or the LT-β receptor. We investigated the humoral immune responses of LT-α deficient mice to orally or foot-pad (fp) administered live HSV-1 KOS. Additionally, we evaluated the ability of HSV-1 specific CTL to kill various antigen presenting target cells. The data presented here support a defect in the generation of antigen specific CTL in LT-α deficient mice. Although these mice generate both mucosal and antigen specific antibodies to HSV following oral and fp administration of virus and expansion of $CD8^+$ T cell numbers following *in vitro* re-stimulation with HSV, they fail to elicit significant CTL responses. These data expand upon the current

knowledge of LT-α function and suggest a critical role for LT-α in the development of functional CTL responses but not in the development of $CD8^+$ T cells.

Chapter 2: Introduction

Mice rendered genetically deficient for the cytokine, lymphotoxin-alpha (LT-α), have been shown to be deficient in mucosal and systemic antibody production following antigen administration by various routes and in the clearance of intracellular parasites (1,2,3). These deficiencies are suspected to be due to a combination of consequences which result from a lack of organized secondary lymphoid tissues, impaired receptor triggering, and impaired signaling event outcomes following appropriate ligand receptor interaction. LT-α deficient (mutant) mice have no alteration in the numbers of circulating T lymphocytes (see Part 2) but mice with impaired signaling at the tumor necrosis factor-α (TNF-α) type 1 p55 receptor (TNRF1) demonstrate decreased clearance of certain parasitic infections which depend on functional cell mediated immunity (4.5). It is unknown what proportion of LT-α interacts at the TNFR1, however this may be the dominant form of the cytokine in adult mice which leads to speculation regarding the involvement of LT-α in the clearance of viral and parasitic infections. We examined the immune responses of LT-α deficient mice to a live replicating virus delivered by two separate routes. Herpes simplex virus (HSV) type 1, strain KOS, was chosen because immunity engendered following infection with this virus is known to be dominated by $CD4^+$ T cells of the T_H1 cytokine profile type and $CD8^+$ cytotoxic T lymphocytes (CTL) (6). Further, this virus has been shown to stimulate viral specific CTL responses in several animal models (7, 8). Surprisingly, although capable of limited specific antibody production which is predominantly of the T_H1 type, LT-a deficient mice show profound impairment in the generation of HSV-1 specific CTL. The data presented here strongly

supports a role for LT-α, either in homotrimeric form interacting at the TNFR1, or in heterotrimeric (LT-α_1:LT-β_2) form interacting at the LT-β receptor, in the generation of viral specific CTL.

Chapter 3: Materials and Methods

Mice

C57BL/6 LT-α deficient (mutant) and wild-type (w.t.) mice used in the experiments described herein were the off-spring of founder mice described previously (see Part 1) and bred to the F8 congenic generation. Mice were placed in the various experimental groups at 5-6 weeks of age and were housed in filter top microisolator cages under sterile conditions throughout the experiments. All procedures described were in compliance with both institutional and IACUC guidelines.

Immunization protocols

Live HSV-1 KOS was used as the immunizing antigen in all experiments described here. Mice receiving oral antigen were starved for 4 hours, anesthetized with inhalant Metofane (Pitman-Moore), and given 100μ of a 1:5 mixture of 7.5% NaH_2PO_4 and 1x HBSS (neutralizing mix) via a 30 gauge orogastric feeding needle 30 minutes prior to antigen administration. Control mice received oral neutralizing mix only. Mice receiving fp immunizations were anesthetized and antigen administered in 30 μl of sterile 1x PBS in both hind footpads. Control mice received only 1x PBS in both hind footpads. The total dose of antigen for each mouse was 5.0×10^6 PFU of live HSV-1 KOS. Viral titers were determined using Vero cell plaque assays. Briefly, virus was grown on Vero cell monolayers at 37 °C with 5% CO_2 until >50% cytopathic effect was achieved (3-4 days). Virally infected cells were collected and freeze thawed three times to lyse infected

cells. The resulting mix was centrifuged at 3000 R.P.M. for 10 minutes to pellet cellular debris and the supernatant was collected and centrifuged further at 10,000 R.P.M. to pellet viral particles. The resultant pellet containing virus was resuspended in 100 µl of sterile 1x PBS and serially diluted two-fold for a total of 7 dilutions. These were layered over uninfected Vero cell monolayers growing in sterile 24-well tissue culture plates, overlaid with sterile 1% low melting point agarose, and incubated as above until obvious plaques were visible within the Vero cell layer (2-4 days). Neutral red dye, 1%, was then layered over the agarose layer and plaques counted. These counts were converted to the viral titer which was expressed as "plaque forming units" (PFU)/ml.

Mice were immunized on Day 0 and Day 7 and were terminated on Day 12 for sample collection.

Sample collection

Prior to each immunization and at termination, blood and feces were collected individually. For fecal slurry preparation, 1 ml of 0.1% sodium azide was added per 100 mg of feces. The samples were vortexed for 15 minutes and then centrifuges at 3000 x *g* for 8 minutes and supernatant collected. Fecal supernatants and sera were stored at -20 °C until analysis.

At the time of termination, spleens and bone marrow cells were collected aseptically as described (see Part 2, Chapter 3). From w.t. mice, popliteal and lumbar lymph nodes (for fp injected mice) or mesenteric lymph nodes (from orally inoculated mice) were also collected. All samples were collected into complete RPMI media and processed to single cell suspensions. Red blood cell depleted samples were then

processed for fluorescence activated cell sorting (FACS) analysis, cytotoxic T cell (CTL) assay, or Yac-lymphoma killing assay.

Antibody determination by Enzyme linked immunosorbent assay (ELISA)

Total and antigen specific IgM, IgG, IgG_1, IgG_{2a} were determined from serum samples and IgA from fecal samples. Coating antibodies were diluted in carbonate buffer, pH 7.9. Standard antibodies and samples were analyzed in triplicate and diluted in 1 x PBS as were secondary and tertiary antibodies. Samples and standards were incubated overnight at 4° C. Non-specific protein binding was blocked with 3% non-fat dry milk in 1 x PBS containing 0.05% Tween 20 and plates incubated for 2 hours at 37° C. Secondary and tertiary antibodies were incubated for 2 and 1 hours, respectively, at 37° C. Plates were washed 3 times with 1 x PBS containing 0.05% Tween 20 between each incubation. **Table 1** (page 184) lists all antibodies used in these studies.

FACS analysis

Suspensions containing 1.0 x 10^6 cells were incubated with 1 μg each of a FITC- and PE- labeled antibody. Combinations included B220 and Thy1.2, CD4 and CD8, and B220 and pan NK cell marker. All antibodies were purchased from Pharmingen (San Diego, CA). Antibodies were incubated with cell populations for 1 hour at room temperature on a rocking shaker. Cells were then washed 3 times with FACS buffer containing 2.5% bovine serum albumin and 0.1% sodium azide in 1x PBS and then were fixed in 4% paraformaldehyde. Cells were analyzed on a Becton-Dickinson FACScan using Cell-Quest Software.

Cytotoxic T lymphocyte (CTL) Assays

Effector cells [collected from spleens, bone marrow, or lymph nodes (from w.t. mice] were analyzed for the presence of antigen-specific MHC-restricted CTLs. The effectors were restimulated in vitro with UV-inactivated HSV-1 KOS [multiplicity of infection (m.o.i.) =1.5] in limiting dilution in complete RPMI media and incubated at 37 °C for 5 days. The effector cells were then mixed at varying ratios with sodium chromate (Cr^{51}) labeled target cells for 4 hours. The targets included EL-4 (H-2^b) and EMT-6 (H-2^d) cells transiently infected with 5 m.o.i. HSV-1 or reovirus 1, strain Lange. Uninfected EL-4 targets were used as syngeneic uninfected controls. The supernatants were collected after the incubation period for determination of chromium release. The results were expressed as **percent specific lysis** = (cpm of sample release – cpm of spontaneous release)/(cpm of total release – cpm of spontaneous release) x 100.

Yac assay: Detection of NK cell function

Yac lymphoma cells in log growth phase were suspended to a concentration of 2.0 x 10^6 cells/ml in complete RPMI and incubated for 90 minutes at 37 °C with 100 μCi of Cr^{51}. Cells were then washed twice in complete RPMI and plated at 2.0 x 10^4 cells per well in a 96-well tissue culture plate. These cells served as targets for NK cell killing. Suspensions of cells collected from various tissues served as effector populations and were added at effector:target ratios of 1:100 through 1:3.1. Effector and target cell populations were incubated at 37 °C for 4 hours after which time supernatants were collected and analyzed for specific lysis as determined by the release of chromium.

Statistics

Where applicable, a Student's two-tailed T-test was utilized to determine significances of differences between groups and significance level was set at $p < 0.05$.

Chapter 4: Results

LT-α deficient mice generate reduced immunoglobulin levels than due w.t. mice regardless of the route of antigen administration:

Table 2 (page 185) displays IgA, IgM, and IgG antibody levels measured from the various groups. Each value represents the mean of values determined from 4 separate experiments. Standard errors of the mean are provided. In all situations, LT-α deficient (mutant) mice generated lower levels of total and HSV specific fecal IgA, serum IgM, serum IgG, serum IgG_1, and serum IgG_{2a}. Interestingly, mutant mice consistently demonstrated dominant IgG_{2a} responses over IgG_1 responses. This was also true for w.t. mice however the IgG_1:IgG_{2a} ratios were less in mutant than they were in w.t. mice (**Table 3**, page 186).

LT-α deficient mice possess similar numbers of peripheral blood lymphocytes as w.t. mice before and after in vitro stimulation

Because the CTL activity of mutant mice following immunization with live HSV-1 was of particular interest, we analyzed spleen and lymph node populations by FACS analysis before and following 5 days of culture with uv inactivated HSV-1 virus. As illustrated in **Table 4** (page 187), mutant and w.t. mice have similar numbers of $CD4^+$, $CD8^+$, $B220^+$, and $Thy1.2^+$ cells when compared to w.t. mice at termination on Day 12 of study. Additionally, as shown in **Figure 1** (page 183) mutant and w.t. mice both demonstrated an increase in the number of $CD4^+$ and $CD8^+$ T cells but a decrease in all

other cell populations following *in vitro* incubation with killed HSV-1. However, when examining for NK cell numbers a difference, although not statistically significant ($p > 0.05$), was noted between mutant and w.t. mice (**Table 4**).

LT-deficient mice demonstrate severely impaired HSV specific CTL function

Specific lysis of syngeneic antigen specific targets, as determined by chromium release assays, revealed that mutant mice, although possessing normal numbers of $CD8^+$ T cells which were capable of proliferation, were unable to generate significant CTL responses following *in vivo* priming and boosting and *in vitro* restimulation with HSV-1 KOS. **Table 5**, (page 188) lists results of cumulative CTL results of both PO and FP immunized mice. Allogeneic killing, as determined both by both MHC and antigen mismatch, was similar in both w.t. and mutant mice and below background levels thereby supporting the specificity of killing observed in w.t. mice.

NK cell killing does not contribute significantly to target cell killing in either w.t. or mutant mice

The percent specific lysis observed following co-incubation of chromium labeled Yac lymphoma cells with cell populations from mutant and w.t. mice was consistently at or below measured background which was determined by the spontaneous release of chromium from Yac cells incubated without effector cell populations. There was no significant difference in any of the Yac killing assays performed in this study between w.t. and mutant cell populations. The viability of Yac cells was confirmed by

microscopic examination of representative cell cultures by visualizing both increases in cell density over time and formation of characteristic cellular aggregations. There was no evidence of viral or bacterial contamination of cell cultures as determined by incubation of Yac culture supernatant on Vero cell monolayers or on Luria-Bertani agar.

Chapter 5: Discussion

The data presented here give strong indication that the genetic deficiency of LT-α in some way affects the development of functional CTL. The detection of antigen specific mucosal and systemic antibodies following both oral and systemic administration of HSV-1 KOS indicates specific antigen recognition and processing by mutant mice. The diminished HSV-1 specific antibody production observed in mutant mice is consistent with previous findings (see previous parts) and is likely a reflection of the lack of secondary lymphoid tissues in mutant mice as well as the nature of HSV-1 KOS as an antigen (9, 10). However, mutant mice possess equivalent numbers of B and T lymphocytes as do w.t. mice, as evidenced by FACS analysis, and T lymphocytes derived from immunized mutant and w.t. mice undergo expansion following *in vitro* re-stimulation with killed virus. Curiously, however, only lymphocytes derived from immunized w.t. mice demonstrated viral specific killing of chromium labeled target cells. This suggests that some form of LT-α triggering is required for the development of the cytotoxic phenotype of $CD8^+$ T cells. The most logical explanation is that failure of triggering through the TNFR1 by homotrimeric LT-α is responsible for the impaired CTL function. This is supported by the knowledge that TNFR1 deficient mice are impaired in their ability to clear intracellular parasite infections (4, 5). However, there is no evidence to support a deficiency in either TNF-α levels or function in LT-α deficient mice (2) and since TNF-α has been reported to be the dominant inflammatory cytokine of the two, this is an unlikely explanation.

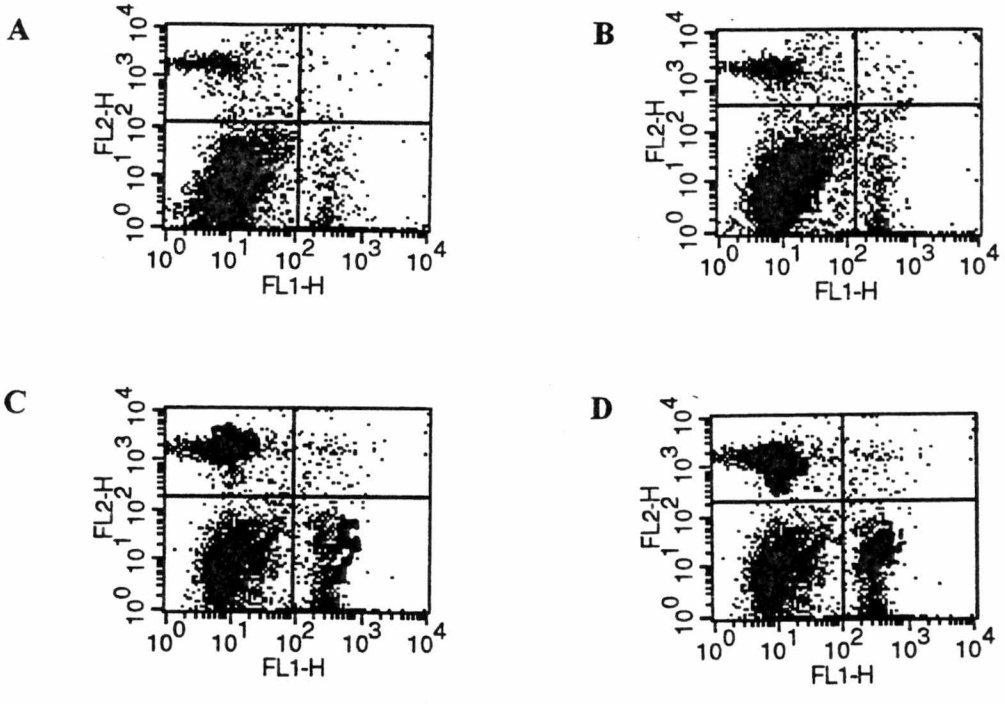
Cytotoxic T lymphocytes and NK cells both express the surface LT-βR which specifically binds LT-α_1:LT-β_2 but not TNF-α (11). The lack of appropriate triggering of the LT-βR receptor would therefor explain impaired CTL and NK killing in mutant mice but not the impaired NK killing observed in w.t. mice. It is possible that there is a necessary interaction between intracellular signaling intermediates of the TNFR1 and LT-βR signaling cascades which leads to the generation of functionally mature CTL. The data presented here would support this idea. Unfortunately, the lack of NK killing observed cannot be explained and may perhaps be simply due to the assay techniques or the Yac cell population utilized.

The CTL data generated from w.t. mice requires further interpretation. Specifically, syngeneic antigen specific killing was quite high and did not demonstrate appropriate dilution. This was, however, observed in all experiments which were evaluated for data presentation. Although this requires further clarification, there is sufficient and convincing evidence for intact CTL triggering in w.t. mice but not in mutant mice following exposure to HSV-1 KOS.

The information provided by these experiments allows a broader interpretation of the consequences of LT-α deficiency on immune function and responsiveness. Mice deficient in this cytokine are capable of mounting specific but impaired humoral immune responses to several antigens delivered by different routes. The antibody isotype profiles generated by immunized LT-α deficient mice support cell mediated involvement in the generation of antibody responses which appear to be dominated by T_H1 type T cells (see **Table 3**) and is consistent with expected immune responses to HSV-1 (7). Lacking in

this study is an assessment of cytokine profiles generated both in immunized mice and in the supernatants of *in vitro* re-stimulated cell populations. Clearly, however, LT-α deficient mice demonstrate an impairment in immune function which involves $CD8^+$ CTLs. There is evidence that LT-α deficient mice do generate appropriate proliferative responses following oral and fp immunization with HSV-1 (data incomplete and not presented). Since proliferation assays reflect antigen specific expansion of $CD4^+$ T cell populations, this would indicate that LT-α deficient mice have no functional impairment of this immune compartment. Unfortunately, this issue also requires further clarification.

Figure 1: Both LT-α deficient and w.t. mice demonstrate increased . $CD4^+$ and $CD8^+$ T cells following *in vitro* restimulation with uv inactivated HSV-1 KOS at an MOI of 1.5. $CD4^+$ cells are in the upper left and $CD8^+$ cells are in the lower right quadrant. The lower left quadrant contains unstained cells.



A = immunized mutant splenocytes, freshly harvested: $CD4^+$ = 18.1, $CD8^+$ = 10.6

B = immunized mutant splenocytes, 5 days restimulation: $CD4^+$ = 37.2, $CD8^+$ = 22.8

C = immunized w.t. splenocytes, freshly harvested: $CD4^+$ = 20.7, $CD8^+$ = 12.1

D = immunized w.t. splenocytes, 5 days restimulation: $CD4^+$ = 35.9, $CD8^+$ = 25.3

Table 1: List of antibodies and reagents used in ELISA assays.

Coating antibody And Dilution	Standard Antibody And Dilution	Secondary Antibody And Dilution	Tertiary Antibody And Dilution
Goat anti-mouse IgM, Jackson, 115-005-075, 1.7 mg/ml, Diluted 1:1000	Mouse myeloma IgM, MOPC-104E, Sigma, M-5170, 1.0 mg/ml, Diluted 1:4000	HRP-rabbit anti-mouse IgM, Zymed, 61-6820, Diluted 1:2000	None
Goat anti-mouse IgG, So. Biotech. Assoc., 1030-01, 1.0 mg/ml. Diluted 1:1000	Mouse IgG- UNLB, So. Biotech. Assoc., 107-01, 0.5 mg/ml. Dilute 1:5000	HRP-goat anti-mouse IgG, So. Biotech. Assoc., 1030-05, Dilute 1:2000	None
Goat anti-mouse IgG_1, So. Biotech. Assoc., 102-01, 1.0 mg/ml. Diluted 1:1000	Mouse IgG_1- UNLB, So. Biotech. Assoc., 102-01, 1.0 mg/ml. Diluted 1.25:5000	HRP-goat anti mouse IgG_1, So. Biotech. Assoc., 1070-05 Diluted 1:2000	None
Goat anti-mouse IgG_{2a}, So. Biotech. Assoc., 103-01, 1.0 mg/ml. Diluted 1:1000	Mouse IgG_{2a}- UNLB, So. Biotech. Assoc., 1030-01, 1.0 mg/ml Diluted 1.25:5000	HRP-goat anti-mouse IgG_{2a}, So. Biotech. Assoc., 1080-05 Dilute 1:2000	None
Rabbit anti-mouse IgA, Zymed, 61-6700, 0.5 mg/ml. Diluted 1:250	Purified Mouse myeloma IgA, Zymed, 02-6500 1 mg/ml Dilute 1:10,000	Biotin-goat anti-mouse IgA, Zymed, 62-6740, Dilute 1:2000	Peroxidase-conjugated streptavidin, Jackson, 016-030-084, 1 mg/ml, Dilute 1:2000.
HSV antigen, Advanced Biotechnology Inc., 10-515-001, Diluted to 2 µg/ml	Mouse IgG- UNLB or Mouse IgG_1- UNLB or Mouse IgG_{2a}- UNLB or Purified Mouse myeloma IgA	HRP-goat anti-mouse IgG or HRP-goat anti mouse IgG_1 or HRP-goat anti-mouse IgG_{2a} or Biotin-goat anti-mouse IgA	None Or Peroxidase-conjugated streptavidin

Table 2: Antibody responses in mutant and w.t. mice following HSV-1 KOS administration by either the PO (oral) or FP (foot pad) route.

Group	Total fecal IgA	HSV:IgA	Total serum IgM	HSV:IgM	Total serum IgG	HSV:IgG
-/- N[1]	2702.2[2] +/-276.0	ND[3]	73173.1 +/- 5209.7	ND	400939.6 +/-52739.8	ND
-/- PO	3699.3 +/-1350.3	2.2 +/-5.4	159473.7 +/- 7226.4	194.3 +/- 11.9	444676.9 +/-193501.1	7.1 +/-4.8
-/- FP	2771.3 +/-523.5	15.7 +/-0.9	124970.5 +/- 8836.4	152.9 +/- 6.2	1010150.2 +/-139760.3	31.2 +/-5.1
+/+ N	478907.7 +/-10047.9	ND	173972.9 +/- 18718.3	ND	1098693.6 +/-177359.2	ND
+/+ PO	880420.4 +/-171125.2	136.2 +/-25.6	170455.8 +/- 10993.2	1577.4 +/- 144.8	1414873.6 +/-32298.2	361.7 +/-200.1
+/+ FP	735698.6 +/-130394.0	72.1 +/-2.2	195846.1 +/- 23263.3	1173.0 +/- 193.6	1342462.9 +/-36580.3	1491.6 +/-508.7

[1] = N indicates naïve mice of either group. No significant difference was found between naïve mice within each genotypic group therefor the value presented is for all naïve mice.

[2] = All values are presented as ng/ml.

[3] = ND indicates that values were not detectable or were below 0.8 ng/ml.

Table 3: Generated total and antigen specific IgG_1:IgG_{2a} ratios following oral or FP immunization.

Group	Total serum IgG_1:IgG_{2a}	HSV:IgG_1:IgG_{2a}
-/- N[1]	1:5.1	ND[2]
-/- PO	1:3.9	1:3.3
-/- FP	1:3.7	1:3.1
+/+ N	1:1.4	ND
+/+ PO	1:2.2	1:2.2
+/+ FP	1:2.0	1:1.8

[1] = N indicates naïve mice of either group. No significant difference was found between naïve mice within each genotypic group therefor the value presented is for all naïve mice.

[2] = ND indicates that values were not detectable or were below 0.8 ng/ml.

Table 4: FACS analysis results comparing populations of lymphocytes in LT-α mutant and w.t. mice.

Sample	%CD4	%CD8	%B220	%Thy1.2	%NK
mutant N[1]	23.7	11.9	47.6	32.7	3.7
mutant PO[2]	25.9	13.2	51.9	36.4	4.6
mutant FP[3]	22.5	14.0	53.1	35.8	4.7
w.t. N	24.6	10.6	45.2	32.7	5.2
w.t. PO_{spleen}	24.9	12.7	46.3	33.2	5.4
w.t. PO_{DLN}[4]	22.7	23.2	36.7	42.6	6.0
w.t. FP_{spleen}	23.9	11.1	47.4	35.1	6.3
w.t. FP_{DLN}[5]	26.4	37.2	39.5	51.2	7.1

[1] = N indicates Naïve mice
[2] = orally administered antigen
[3] = foot pad administered antigen
[4] = draining lymph nodes include mesenterics
[5] = draining lymph nodes includes popliteals and lumbars

Table 5: Percent specific lysis as measured by chromium release is severely impaired in LT-α deficient mice.

Group	% Specific Lysis at various Effector:Target ratios				
	100:1	50:1	25:1	12.5:1	6.3:1
-/- N^{1}_{BM}[2]	11.2	15.5	9.7	4.3	5.8
-/- N_{spleen}	7.0	4.5	4.8	5.1	4.8
-/- I_{BM}	4.8	5.1	4.7	3.9	3.3
-/- I^{3}_{spleen}	9.3	7.7	7.2	4.4	1.6
+/+ N_{BM}	12.4	11.3	8.7	5.5	4.9
+/+ N_{spleen}	2.3	4.8	4.5	5.1	4.6
+/+ N_{DLN}[4]	8.0	9.2	7.8	5.8	4.7
+/+ I_{BM}	87.2	83.0	78.2	69.6	62.7
+/+ I_{spleen}	55.9	79.9	65.4	60.6	56.8
+/+ I_{DLN}	43.7	46.3	39.7	37.6	42.5

[1] = N indicates naïve mice
[2] = bone marrow
[3] = I indicates immunized mice, only data from orally immunized mice is presented.
[4] = draining lymph nodes indicates mesenteric nodes.

Literature cited

1. De Togni, P., Goellner, J., Ruddle, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. S., Carlson, R., Shornick, L. P., Strauss-Schoenberger, J., Russell, J. H., Karr, R., and Chaplin, D. D. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264: 703-707.
2. Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D., M., Thomas, J., Kanangat, S., and Mucenski, M. L., 1995. Lymphotoxin-α-deficient mice: Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155: 1685-1693.
3. Matsumoto, M., Fu, Y.-X., Molina, H., and Chaplin, D. D. 1997. Lymphotoxin-α-deficient mice and TNF receptor-1-deficient mice define developmental and functional characteristics of germinal centers. *Immunol. Rev.* 156: 137-144.
4. Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. 1993. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes. Nature* 364: 798-802.
5. Castinos-Velez, E., Maerlan, S., Mosorio, L., Aberg, F., Biberfeld, P, Orn, A., and Rottenberg, M. C. 1998. *Trypanosoma cruzi* infection in tumor necrosis factor receptor p55-deficient mice. *Infect. Immun.* 66(6): 2960-2968.
6. Manickan, E., Francotte,, M., Kuklin, N., Dwarchin, M., Malitor, C., Gheyson, D., Slauoi, M., and Rouse, B. T. 1995. Vaccination with recombinant vaccinia virus

expressing ICP 27 induces protective immunity against herpes simplex virus through $CD4^+$ $Th1^+$ T-cells. *J. Virol.* 69: 4711-4716.

7. Manickan, E., Yu, Z., Rouse, R. J. D., Wire, W. S., and Rouse, B. T. 1995. Induction of protective immunity against herpes simplex virus with DNA encoding the immediate early protein ICP 27. *Viral Immunol.* 8(2): 53-61.
8. Kanangat, S., Thomas, J., Gangappa, S., Babu, J. S., and Rouse, B. T. 1996. Herpes simplex virus type 1-mediated up-regulation of IL-12 (p40) mRNA expression: implications in immunopathogenesis and protection. *J. Immunol.* 156: 1110-1116.
9. Rouse, B. T. and Wagner, H. 1984. Frequency of herpes simplex virus-specific cytotoxic T lymphocyte precursors in lymph node cells of infected mice. *Immunology.* 51: 57-64.
10. Wagner, H., Hardt, C., Rouse, B. T., Rollinghoff, M., Scheurich, P., and Pfizenmaier, K. 1982. Dissectionof the proliferative and differentiative signals controlling murine cytotoxic T lymphocyte responses. *J. Exp. Med.* 155: 1876-1881.
11. Crowe, P. D., VanArsdale, T. L., Walters, B. N., Ware, C. F., Hession, C., Ehrenfels, B., Browning, J. L., Din, W. S., Goodwin, R. G., and Smith, C. A. 1994. A lymphotoxin-β-specific receptor. *Science* 264: 707-709.

Part 7: Concluding Remarks

The phenotype of the LT-α deficient mouse was quite surprising given that the majority of mediators of the immune system are considered to be redundant. Nevertheless, this cytokine deficient mouse has proven to be a useful tool in understanding which components of the immune system are necessary for the generation of immune responses and which are dispensible. Specifically with respect to the mucosal immune system, the currently embraced dogma is that Peyer's patches and mesenteric lymph nodes are absolutely essential for the generation of antigen specific IgA responses against antigen encountered across the gut epithelium. Certainly, this is not true as evidenced by the data presented in this dissertation. However, this is a highly contrived system since LT-α deficient mice used in these studies were maintained under near sterile conditions and when exposed to foreign antigen, only one such antigen was administered at a time. This certainly is not the case in the "real" world. Nonetheless, by studying the complete phenotype of the LT-α deficient mouse and by utilizing various reconstitution models, the components essential for mucosal immune responses and their ability to compensate for the loss of other immune compartments can be scrutinized.

Therefore, based on the studies presented here, it can be concluded that the developmental absence of LT-α does not affect vascular endothelial expression of select adhesion molecules nor does it affect the development of various lymphocyte populations. LT-α deficient lymphocytes are not impaired in their homing ability nor are they functionally impaired in responses to T dependent antigens. Finally, neither Peyer's patches, mesenteric lymph nodes, nor the spleen are essential for the development of mucosal immune responses to orally administered antigen. On the other hand, LT-α is

critical for the development of organized lymphoid tissues and therefore the generation of optimal immune responses to antigens delivered by various routes. Additionally, LT-α signaling appears to be critical for the generation of functional antigen specific $CD8^+$ cytotoxic T lymphocytes.

The molecular mechanisms involved in the events which are exclusive to and specifically require LT-α are not yet defined. It is likely that specific receptor interactions, some currently identified and others not, will play major roles. Regardless, the LT-α deficient mouse provides a useful toll for determining the necessity of organized lymphoid tissues in certain immune inflammatory conditions such as herpes stromal keratitis and inflammatory bowel disease. The former condition specifically requires $CD4^+$ T cells which become primed in the cervical lymph nodes. The latter condition may be regulated by certain intraepithelial and lamina propria cell populations. Use of the LT-α deficient mouse model in these and other disease states may provide clues to disease control or perhaps prevention. Minimally, this mouse model may provide insight to certain human immune deficient states.

Vitae

The lives of some people are fascinatingly told

From their nacent arrival to when they are old.

Perhaps it's a curious name or an interesting look

That gets some folks' lives recorded in books.

What a shame a birth right isn't instantly given

And so many of us must be constantly driven

To live up to some standard set unfairly by others

And walk away from the path worn by fathers and mothers.

So while I give thanks, I receive no praise

Since it's disrespectful to not follow the old ways.

But as I walk along this red road of my life

And regardless of the hunger and pain and strife,

In the end I'll find comfort as I reflect on the choices,

Made with hesitation but not without the voices

Of those gone before to illuminate my path

So that I will know when the goal is reached at last

That it was me all along, I found my own way.

I am responsible for who I am today.

- Ila Anne Davis, 6/98

Ila Anne Davis received a Bachelors of Science Degree in Molecular Biology from San Jose State University, San Jose, California, in 1985. She attended The University of

California at Davis, College of Veterinary Medicine, and graduated with Honors and a Doctorate of Veterinary Medicine in 1989. She married Eric Davis the day after graduation and moved back to her home town of Fallon, Nevada, just one week later to become the first female veterinarian to practice in Central Nevada. In 1990, she began internship training in large animal medicine at Iowa State University, Ames, Iowa and extended her time in Iowa to complete an ACVIM approved residency followed by a year-long Clinical Instructorship position. She arrived in Tennessee in 1994 and began a Ph.D. program in Immunology in the laboratory of Dr. Barry Rouse in January 1995. Her interest in mucosal immunology stems from her interest in enteric diseases of equine, camelid, and bovine neonates. Dr. Davis is ACVIM board qualified in internal medicine and will complete board certification in 1999. She hopes to some day teach at a veterinary college.