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An ENU-induced mutation in the lymphotoxin α gene impairs lymph organogenesis of lymphoid tissues in C57BL/6 mice

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Abstract

The TNF family is critical for development of lymphoid organs and plays significant roles in regulation of innate and adoptive immune responses. Here we describe a novel N-ethyl-N-nitrosourea (ENU)-induced mutant strain, HLB382, with a point mutation in the *Lta* gene, which encodes lymphotoxin (LT) α , a member of the TNF family. Mutant mice had no lymph nodes and no Peyer's patches. Microscopically, the spleens had disordered follicles and no germinal centers or discernible marginal zones (MZ). While the development of T cells and follicular B cells was normal, the numbers of NK and MZ B cells were significantly reduced. Genomic DNA sequences revealed a single base pair insertion in the coding region of *Lta* resulting in a frame shift and a premature stop codon. This new strain provides opportunities for understanding the full range of *Lta* gene function on a pure C57BL/6 background.

Keywords

lymph organogenesis; lymph node; spleen; marginal zone; leukocytosis; B1b; bone marrow; thymus; ENU

INTRODUCTION

Signaling mediated by the TNF family of cytokines and their receptors is critical for the histogenesis of peripheral lymphoid tissues. Mice with engineered mutations in *Tnf*, *Lta*, *Ltb*, *Libr*, *Tnfrsf1 α* (TNF-R1), *Tnfrsf11 α* (RANK) and *Tnfrsf11* (RANKL) all exhibit changes in the presence and/or number of peripheral and mesenteric lymph nodes (LN) and Peyer's patches, the segregation of T and B cell zones in the spleen, the organization of the splenic marginal zone (MZ), the presence of primary B cell follicles and the presence of follicular dendritic cell (FDC) networks (reviewed in [1]). Insights into how the products of these genes exert their effects are sometimes complicated by unexpected variability in the phenotypes of mice with engineered null alleles of the same locus. For example, Peyer's patches were present in some lines of *Tnf* knockout mice [2;3] but were completely lacking in another [4]. Understanding these differences is complicated by the fact that the *Tnf*, *Lta*, and *Ltb* loci are closely linked on chromosome 17. As a result, gene targeting events directed at one of these loci could alter the physiologic patterns of expression displayed by the others. Alternatively, the mutations may

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unknowingly have affected the activity of other genetic elements, such as miRNAs, that reside within the deleted regions. Since the engineered mutations have commonly been studied on mixed genetic backgrounds often involving strains 129 and C57BL/6 (B6), it is also possible that the differing phenotypes could be due to epistasis. Finally, other studies have shown that altering the expression of one component of the *Tnf/Tnfr* and *Lta/Ltb-Ltbr* signaling pathways may affect complementary or synergistic interactions with another [5].

Most of these potential complications would be obviated if mice with disabling point mutations in each of the linked loci could be identified. Here we describe an ENU-induced mutant strain with a point mutation in the *Lta* gene. Availability of this mutant strain on a pure B6 background should enable definitive studies of the roles played by *Lta* in the development and function of the immune system and in various disease conditions.

MATERIALS AND METHODS

Animals

C57BL/6J (B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). A mutant B6 mouse, HLB382, was generated as part of an ENU project in the Jackson Laboratory Center for Mouse Models of Heart, Lung, Blood and Sleep Disorders and found to have leukocytosis (<http://pga.jax.org/about/index.html>). The heritability of the phenotype was confirmed. The third generation of homozygous mice was used in this study. The use of mice in this study was approved by NIAID Animal Care and Use Committee under the protocol LIP-4.

Histological analysis

Tissues collected from liver, lung, kidney, thymus, spleen and LN including inguinal, cervical and mesenteric nodes, and intestine were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). In some experiments, frozen splenic samples were processed for immunohistochemical staining with antibodies against IgM (Southern Biotech, Birmingham, AL) and BCL6 (Santa Cruz Biotechnology, Santa Cruz, CA) using standard procedures as previously reported [6].

Flow cytometric (FACS) analyses

Single cell suspensions prepared from primary and secondary lymphoid compartments were analyzed by FACS as described previously [7]. Antibodies for phenotyping were obtained from BD-Biosciences (San Diego, CA). Cells were analyzed using a FACSCalibur (Becton Dickinson, Mountain View, CA).

Mutation mapping

Genotyping to identify the genomic location of HLB382 was performed by the Jackson Laboratory Scientific Services' Fine Mapping Laboratory using an F2 intercross with C3H/HeJ mice. The mutation was mapped to an interval of chromosome 17 bounded by *D17Mit175* and *D17Mit51*.

Identification of the *Lta* mutation

Genomic DNA was prepared from the tails of B6 and HLB382 mice. PCR primers for cloning were: *Lta* sense, 5'-CCCTGTCTCCTTGGGCTTA-3'; *Lta* antisense, 5'-AGGAAGTTCTAGGTAGGCTTGA-3'. Amplicons were cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced by the Research Technology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Hamilton, MT) and Bionexus (Oakland, CA).

For cDNA cloning of LT β and TNF, total RNA was extracted from spleens of HLB382 and B6 mice and reverse transcribed using a ThermoScript RT-PCR system (Invitrogen, San Diego, CA). The cDNA was cloned and sequenced as described above. PCR primers for cloning were: *Ltb* sense, 5'-ATGGGGACACGGGGACTG-3'; *Ltb* antisense, 5'-ATTCGTGTCCTACATTCCGTTC-3'; *Tnf* sense, 5'-AAAGCAAGCAGCCAACCAG-3'; *Tnf* antisense, 5'-GGTCACTGTCCCAGCATCTTG-3'.

Analysis of LT α protein expression

Splenic cells from HLB382 and B6 mice were cultured overnight with and without 20 μ g/ml of lipopolysaccharide (LPS) (InvivoGen, San Diego, CA), 1 μ g/ml of CpG oligodeoxynucleotide (ODN) 1826 (InvivoGen) or 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO). The cells were stained with a hamster anti-LT α antibody (BD-Biosciences) followed by biotin-labeled anti-hamster IgG and streptavidin-conjugated PE and were analyzed by FACS.

Measurement of TNF

HLB382 and B6 mice were injected intraperitoneally (i.p.) with 100 μ g of LPS or PBS as a control. After 2 h, the mice were bled from the tail, and serum TNF levels were quantified by ELISA using a Mouse TNF- α Ready-SET-Go ELISA Kit (eBioscience, San Diego, CA).

RESULTS AND DISCUSSION

Identification of mutant HLB382 and genetic mapping studies

White blood cell (WBC) counts performed as part of the screening of third-generation mutagenized mice identified a phenotypic deviant with a significantly increased white blood count on non-fasted whole blood analysis (WBC = 15.39×10^3 cells/ μ l).

The 382 mutation was mapped to mouse chromosome 17 between 17.7 and 24.5 cM. An analysis of genes lying within this interval suggested *Lta* as a candidate when we compared the phenotype of the mutant with those of *Lta* knockout mice (Table 1). Sequencing of *Lta* genomic DNA from the mutant mice revealed insertion of a cytidine between nucleotides 16 and 17 of the coding sequence in the second exon (Fig. 1A). This resulted in a frame shift from amino acid 6 onward and translation of a theoretical new peptide of 173 amino acid (the wild type peptide is 202 amino acid) due to a premature stop codon in Exon 4 (Fig. 1A). Almost all reported ENU-induced mutations are point mutations resulting in single base changes [8]. The insertional mutation identified here is rare but other insertions were identified in *hprt* mutants of ENU-treated monkey T cells [9].

Because *Lta*, *Ltb* and *Tnf* are closed linked and their deletions yield somewhat similar phenotypes, we performed cDNA sequencing of *Ltb* and *Tnf* to rule out the possibility that the phenotype of HLB382 resulted from mutations in *Ltb* or *Tnf* alone or in combination with the mutation in *Lta*. Both HLB382 and B6 mice express equal levels of *Ltb* and *Tnf* transcripts in their spleens (data not shown). In addition, studies of several independent clones showed that the sequences of cDNAs for *Ltb* and *Tnf* of HLB382 and B6 mice were identical.

The probability that there is another coding region mutation within the identified 11 Mb region was estimated to be small ($p=0.033$) using a mutation rate of 1 per 1.1 Mb [10] and the equation of Keays et al. [11] (<http://www.well.ox.ac.uk/enuMutRat>). Thus, the point mutation of *Lta* may be a single molecular event.

Lack of *Lta*-encoded protein expression in HLB382 mice

LT α forms complexes with LT β on the cell surface of activated B and T cells [12]. To determine whether expression of LT α protein was affected in HLB382 mice, we stimulated HLB382 and B6 splenic B cells with LPS, PMA and CpG ODN to enhance LT α expression. FACS analysis of B220⁺ B cells showed that expression of LT α protein was enhanced on B6 but not on HLB382 cells stimulated with either LPS or PMA. Stimulation with CpG ODN had no effect on cells of either strain (Fig. 1B). The staining seen on HLB382 cells was at background levels and was not considered to be significant. The failure of anti-LT α antibody to stain HLB382 cells is consistent with the sequence data which indicated that the mutated gene would produce an immunologically unrelated protein.

The *Lta* mutation did not affect LPS-induced systemic TNF response in vivo

Four independent *Lta* knockout strains have been described (Table 1). Some “conventional” *Lta* knockout mice exhibited impaired TNF production in response to LPS or *Mycobacterium bovis* BCG in vivo [13;14;15]. To determine whether the ENU-induced mutation of *Lta* would affect TNF secretion, we measured serum TNF levels following LPS injection. Consistent with the cDNA sequencing data that revealed no mutations in *Tnf*, the levels of TNF in sera of LPS-injected HLB382 and B6 were comparable (Fig. 1C). Similar results were obtained using the *Lta*^{-/-} strain generated by Liepinsh et al. but differed from data obtained with the strain generated by De Togni et al. [15]. These results reinforced the idea that *Lta* mutation in HLB382 mice had no effect on expression of other neighboring genes *Ltb* and *Tnf*.

Mutant mice exhibit abnormalities of secondary lymphoid organs

At necropsy, none of 12 HLB382 mice analyzed had identifiable peripheral LN (popliteal, inguinal, cervical, axillary) or mesenteric nodes (MLN), and no Peyer’s patches. The sizes and weights of spleens from normal and HLB382 mice were similar. Histological analysis of spleens from mutant mice revealed an absence of both marginal zones and germinal centers (Fig. 2 A and B). In addition, the normal separation of T and B cell zones was disrupted in follicles of mutant mice (Fig. 2B). Furthermore, accumulations of small lymphocytes were observed in the livers and lungs of mutant but not of normal B6 mice (Fig. 2A). Finally, the thymuses of mutant mice were histologically normal (data not shown).

Some of the morphological features of HLB382 mice were different from those of conventionally generated *Lta* null mice (Table 1). First, rare MLN were seen in mutant strains generated by De Togni et al. [16] and Banks et al. [17]. Second, the mice generated by Banks et al. and Liepinsh et al. exhibited splenomegaly [15;17], which was not seen in HLB382 mice. Analyzing a large number of mice would help to clarify some of these issues.

ENU mutant mice exhibit normal T cell development but impaired development of NK cells, dendritic cells and granulocytes

FACS analyses showed that the frequencies of dendritic cells, granulocytes and macrophages were moderately decreased in spleens of HLB382 mice as compared with B6 mice (Fig. 3 A, B and E). The size of the NK cell population in spleens of mutant mice were reduced 62% ($p<0.05$) (Fig. 3 C and E). In mutants, the distribution of CD4⁺ and CD8⁺ T cell subsets was normal in both the thymus and the spleen (Fig. 3D and E). Together, these analyses indicated that the mutation had little effect on development of T cells but resulted in impaired NK cell development, either by affecting the differentiation of NK cells or by altering the recruitment of NK cells to the spleen.

ENU mutant mice exhibit normal B cell development in bone marrow (BM) but lack marginal zone (MZ) B cells in the spleen

FACS analysis revealed that the sizes of bone marrow B cell compartments were indistinguishable for HLB382 and B6 mice (Fig. 4A and data not shown). In the spleen, the frequency and total number of B cells in HLB382 mice were equivalent to B6 12 controls (Fig. 4B). However, cells with a MZ B cell phenotype were almost completely missing in HLB382 mice (Fig. 4B). The absolute numbers of transitional B cells (depicted as T1 and T2 in Fig. 4B) were also moderately reduced (Fig. 3E). These data suggest that the mutation specifically affected MZ B cell development, consistent with the disrupted MZ structure of the follicle in the spleen.

The development of peritoneal B1 cells is altered in ENU mutant mice

The total cell counts from peritoneal washouts were increased more than 2-fold in HLB382 mice compared to B6 controls ($5.1 \pm 0.3 \times 10^6$ vs. $2.2 \pm 0.2 \times 10^6$ for mutant and B6 mice, respectively, $p < 0.001$, $n=4$). While the proportions of B1a cells were reduced, their absolute numbers were comparable to those of B6 mice. However, both the percentage and the absolute number of B1b cells were increased dramatically in mutant mice (Fig. 4C and D) (1.8×10^6 per washout in mutant mice vs. 3×10^5 per washout in B6 mice; $n=3$). The number of B2 cells in the peritoneum of mutant mice was also slightly increased (Fig. 4D).

The other *Lta* null strains were not studied for their B1b cells [15;17;18]. A similar increase in B1b and a decrease in B1a cells was observed in *Tnf* and *Lta* double knockout mice [19]. It will be interesting to verify this finding in other *Lta*^{-/-} mice. How LTα selectively suppresses B1b cell differentiation is currently unclear and warrants further investigation.

Previous studies of four strains with targeted null mutations in *Lta* gave generally similar results but had some inconsistencies (Table 1). The reasons for these discrepancies are currently unknown. Because the genes encoding LTα, LTβ and TNF are tightly clustered within a 12-kb span within the major histocompatibility complex gene complex [20;21;22], gene targeting events directed at one of these loci may have interfered with expression and function of the other closely-linked genes. Except for the mutant strain generated by Liepinsh et al., all other three null strains harbor linked neomycin resistance gene (*Neo*^r) used for selection of engineered ES cells (Table 1). Insertion of the *Neo*^r gene into different sites of a given locus has been found to result in profound effects on expression of genes located as far as 100 kb upstream of the insert [23]. Liepinsh et al. proposed that the neo cassette within the LTα gene may have destroyed the far upstream promoter element essential for TNF expression in macrophages leading to impaired TNF production [15]. Differing from those mutant strains bearing the *Neo*^r gene, HLB382 mutants expressed normal levels of TNF and exhibited no mutation of both *Ltb* and *Tnf* (Fig. 1). Thus, this novel strain is apparently advantageous over engineered *Lta* null strains for determining the functions of LTα.

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Fig. 1.

Identification of point mutation in *Lta* and protein expression of LTα and TNF. (A) The highlight indicates a cytosine insertion in *Lta* Exon 2 of the HLB382 genome. (B) Lack of LTα proteins in HLB382 B cells. Splenic B cells were stimulated with LPS, PMA or CpG ODN overnight and stained with an anti-LTα antibody. The cells were analyzed by FACS. The numbers are percentages of cells falling in each gate. Data are representative of two independent experiments. (C) HLB382 mice elicited normal systemic TNF responses to LPS. HLB382 and B6 mice were injected i.p. with 100 μg of LPS or PBS as a control. Two hours after injection, the mice were bled and the serum TNF levels were measured by ELISA. Each circle represents a mouse.

Fig. 2.

Comparative histology of B6 and HLB382 mice. (A) The livers and lungs of mutant mice exhibited lymphoid infiltrates comprised primarily of lymphocytes that were not found in B6 mice. The spleen of a mutant mouse exhibited disordered follicles and lacked a marginal zone. Tissues were stained with H & E (magnification, x40 for liver and lung, and x20 for spleen). (B) Immunohistochemical analyses of spleens from B6 and HLB382 mice. Staining for BCL6 revealed prominent germinal centers in B6 but not HLB382 spleens. Staining for IgM showed follicles with intermingled B cells and T cells and no discernible marginal sinus or marginal zone in HLB382 mice (Magnification, x40).

Fig. 3.

FACS analysis of spleen and thymic cells. Spleen cells from B6 and HLB382 mice were stained with the indicated antibodies to identify dendritic cells (A), granulocytes and macrophages (B), NK cells (C), and T cells (D). The numbers are percentages of cells fall in each gate. Cells were gated on B220⁺CD3⁻ (B) and CD19⁺CD3⁻ cells. DC, dendritic cells; G, granulocytes; M, macrophages. (E) Frequency of indicated cell types in the spleens of B6 and HLB382 mice. Each population was defined by gating depicted in Fig. 3A–D and Fig. 4. FO, follicular; MZ, marginal zone; Tr, transitional. Gr1⁺ cells included both granulocytes and macrophages. Tr B cells included transitional 1 (T1) and 2 (T2) subsets showed in Fig. 4B. Data are means $\pm$ SEM of 4–5 mice. * p <0.05; ** p <0.0001.

Fig. 4. FACS analysis of B cell development. Cells from bone marrow (A), spleen (B) and peritoneum (C) of B6 and mutant mice were stained and analyzed by FACS. The numbers are percentages of cells falling in each gate. Cells were gated on lymphocytes (A and B) and CD19⁺ cells (C). (D) Frequency of B cell subpopulations in the peritoneum. Data are means $\pm$ SEM of 4 mice per group. * $p < 0.05$.

Phenotypes of HLB382 and LT α knockout mice

Reference	This study	De Togni et al., [24]	Banks et al., [17]	Sean Riminton et al., [18]	Liepinsh et al., [15]
Genomic clone ES cells Strain Neo ^c cassette LN	NA	BALB/c	B6	B6	NR
	NA	129	129	B6	129
	B6	B6;129	B6;129	B6	B6;C3H;129
	NA	+	+	+	Deleted
	-	-	-	-	-
Peyer's patches Spleen	Popliteal	-	-	-	-
	Inguinal	-	-	-	-
	Para-aortic	-	-	-	-
	Axillary	-	-	-	-
	Cervical	-	-	-	-
Peritoneum Blood	Mesenteric	Rare (2%) [16]	Frequent (28.6%)	-	-
	-	-	-	-	-
	Abnormal	Abnormal	Abnormal, occasional splenomegaly	Abnormal	Abnormal splenomegaly
	Normal	Increased by 2-3 fold	Increased in mice with splenomegaly	Increased by 2-fold ^d	NR
	Normal	Slightly reduced	NR	NR	NR
Thymus Bone marrow Lung/liver	Total T cell numbers	Normal	Normal	NR	NR
	CD4/CD8 T cell ratio	Decreased ^b	NR	Decreased ^c	NR
	NK cell numbers	Increased 30%	Normal	NR	NR
	B cell numbers	-	-	-	NR
	MZ B cell numbers	Increased WBC count	NR	NR	NR
Peritoneum Blood	WBC	Increased	Increased in mice with splenomegaly	Increased	NR
	T cells	ND	NR	Increased by 2-fold ^d	NR
	B cells	ND	Normal	Increased by 5-fold ^d	NR
	-	Normal	Normal	Normal ^d	NR
	Normal	NR	Normal	Normal ^d	NR
Thymus Bone marrow Lung/liver	Lymphocyte infiltration	NR	Lymphocyte infiltration	NR	Perivascular lymphocytic infiltrates
	-	-	-	-	-

ND, not determined; NR, not reported; NA, not applicable.

[†]Personal communication with Heinrich Korner (James Cook University, Australia).

^b Referred from Ito et al. [25].

Referred from Smyth et al. [26].

^dReferred from Grech et al. [27].