Chapter 9

ENU Mutagenesis in Mice – Genetic Insight into Impaired Immunity and Disease

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Additional information is available at the end of the chapter

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1. Introduction

Over the last decade biomedical research has seen tremendous advancements in the field of genetics that enables unlimited access to >60 vertebrate genomes—including the human and mouse genomes, two of the most widely studied species in biomedical research. These advancements are largely due to rapid development of high throughput sequencing technologies such as next-generation sequencing (NGS) technologies that allow for more affordable and efficient sequencing compared to traditional Sanger technology. The availability of the entire human genome sequence has accelerated our efforts to gain insight into the genetics underlying human disease. Such efforts include Genome-Wide Association Studies (GWAS) — a widely used approach that examines the association between common genetic variants and specific human disease traits. GWAS has led to the successful identification of a large number of SNPs that are linked with chronic diseases ranging from Crohn's disease, systemic lupus erythematosus (SLE), type I diabetes (T1D), and many other common western world diseases(reviewed by Visscher et al.[1]). On the other hand, genetic deficiencies that cause severe disease—such as primary immunodeficiency diseases associated with a poor survival— represent mostly rare mutations within the human population[2]. Such patients can be found in pediatric clinics and more often than not, the genetic deficiencies underlying disease remain elusive. The availability of NGS, however, offers exciting new opportunities in that it enables the identification of all genome-wide variants in individual patients for limited costs. Nonetheless, both approaches are faced with a significant challenge to identify the causal variants. First of all, most GWAS identify loci that contain more than one SNP but more importantly, SNP maps are incomplete and require in depth probing of the identified genetic region (reviewed by Visscher et al.[1]). Thus the approach is generally not limited to a single SNP, but rather uncovers multiple gene candidates for a single locus and researchers are often left with the critical question to identify variant causality. This is

further complicated by the fact that GWAS is often used for the analysis of complex polygenic traits where gene variants need to exist in combination with one other to assert an effect. In the case of monogenic traits underlying severe disease phenotypes, linkage analysis is rarely an option, whereas whole genome sequencing likely results in the identification of numerous "unique" variants. The biological consequences of such variants would again need to be confirmed and candidate gene selections are guided by *a priori* knowledge of gene function. Thus the challenges have rather shifted from identifying genetic changes to understanding gene-function and identifying gene causality.

Providing insight into the functional genome is not just limited to understanding gene or protein function, but also includes gene regulation and complex interactions with other genes within the context of cellular or organismal function. The mammalian genome is believed to consist of ~22,000 annotated genes—most of which have been poorly described. In addition, there is almost an unlimited number of phenotypes to be probed, making this an even more daunting task. Nonetheless, experimental models, including fruit fly and mouse models, have been extremely valuable in revealing unique insight into gene function. Typically, forward and reverse genetic approaches have been applied in parallel to uncover gene function. Reverse genetics begins with the creation of a genetic change and ends with the identification of a phenotype. This approach is hypothesis-based and assumes a specific gene function up front. On the other hand, forward (or classical) genetics proceeds from phenotype to the identification of a causal genetic change (SNP or mutation). This approach has led to important discoveries in the field of immunology most notably the identification of TLR4 as the sole LPS receptor[3]— a discovery recently awarded with the Nobel Prize. Until a few years ago, identification of such genetic variants required positional cloning. This was once considered an arcane art, requiring significant effort, time and financial resources. However, the current availability of the genome sequence for most inbred mouse strains has eliminated the need for contig construction and trivialized the identification of informative markers for high-resolution mapping and/or the identification of existing variants within an associated chromosomal region. Moreover, low cost high-throughput DNA sequencing has accelerated the process of finding unique mutations either introduced spontaneously or by following treatment with mutagens. The current limitation for forward genetics is rather the restricted number of strong monogenic phenotypes, something also referred to as the "phenotype gap"[4]. To overcome this limitation, germline mutagenesis— in which random mutations are introduced in spermatogonial stem cells— has proven to be an effective approach to expand the number of phenotypes.

2. N-ethyl-N-nitrosourea mutagenesis

In mice, a widely used mutagen to create and expand the number of phenotypes is the alkylating agent N-ethyl-N-nitrosourea (ENU). ENU is a powerful mutagen that according to our latest estimates can introduce more than 3 base-pair changes per million base-pairs of genomic DNA[5]. ENU introduces point mutations in spermatogonial stem cells, predominantly affecting A/T base pairs (44% A/T→T/A transversions and 38% A/T→G/C

transitions), whereas at the protein level, ENU primarily results in missense mutations (64% missense, 26% splicing errors and 10% nonsense mutations)[6]. With three bp changes per million bps and a total length of ~2,717 Mb for the mouse genome, one can calculate that each G1 male carries ~8,000 bp changes genome-wide. With the coding region being 1.3% of total genomic sequence and 76% of random bp changes creating a coding change, it follows that each G1 mouse carries about 80 coding changes genome-wide, according to our latest estimates. These exist in a heterozygous form and do not necessarily cause a phenotype. In our experience, the majority of ENU-induced mutations, behave as recessive traits or are codominant at best. The approach entails a weekly injection of ~90mg/kg ENU for 3 weeks that is followed by a brief period of sterility for up to 12 weeks. After the recovery period, each G0 male is bred to untreated, wild type C57BL/6 female mice to generate G1 offspring. These G1 animals are then either used for phenotypic screens or can be used to produce G2 mice, which in turn are backcrossed to the G1 male to generate G3 offspring. While screening the G1 population for phenotypes is limited to the identification of dominant mutations, screening of G3 mice allows for the discovery of recessive mutations. Although the total number of base-pair changes in G3 mice will be reduced—each mouse will carry ~11 coding changes in homozygous form—this has proven to be the more powerful approach to capture mice with phenotypes of interest and more importantly allows for the retrieval of lethal phenotypes.

The rate-limiting step in ENU mutagenesis has long been the identification of causative mutations. Until recently, identified mutant lines were outcrossed to genetically different inbred strains and often the analyses of hundreds if not thousands of meiosis were needed to obtain a small enough critical region that could be sequenced. However, the availability of NGS has significantly facilitated the process of variant identification. Currently, targeted exon-enrichment—i.e. targeting exonic sequence within a critical region using sequence capture probes—, whole-exome and whole-genome sequencing are all proven strategies to effectively uncover mutations. The coverage of (targeted) genomic DNA is often exceptional, particularly for the exon-enrichment approach, where generally high quality sequence (minimal depth >10) for more than >98 % of the targeted region can be obtained[5]. Nonetheless, causality of the identified mutations remains a critical aspect of this approach and low resolution mapping (generally < 30 meioses) and/or genetic confirmation are still integral parts of the ENU mutagenesis approach. In addition, the availability of NGS also provides further opportunities for the phenotypic probing of ENU germline mutants. Often, phenotypes identified in ENU germline mice are lost or significantly influenced by modifier loci located on outcross strains carrying a high degree of genetic variation. For example, identification of genes required for optimal NK cell function has been difficult because of the large variation in NK cell ligands/receptors existing on different mouse backgrounds (Hoebe, unpublished results). By being able to analyze and sequence large genomic regions, fine mapping is superfluous and the exploration of subtle phenotypes can be traced following an outcross to strains with minimal genetic variation between the outcross and parent ENU strain. Ultimately, the genetic diversity should be just enough to allow low-resolution linkage analysis—a prime example being the genetic diversity between C57BL/6J and C57BL/10J strains.

3. Unraveling lymphocyte immune function using ENU mutagenesis

As referred to above, a critical aspect of ENU mutagenesis is the (biological) field of interest to be probed. ENU mutagenesis has been used to define the genetic footprint of a wide variety of phenotypes, including visible, behavioral, developmental and immunological phenotypes[7]. Nonetheless, its success is depending on: 1) the use of reliable screening assays with limited biological variation, 2) targeting large genomic footprints, and 3) probing a biological phenotype that is poorly defined. Our laboratory has used ENU mutagenesis to identify genes with non-redundant function in lymphocyte development, priming or effector function. Among the biological screens we apply is an *in vivo* cytotoxicity assay in which we test the ability of G3 mice to induce an antigen-specific $CD8^+$ T cell response following immunization with irradiated cells containing antigen. In parallel, we test the ability of Natural Killer (NK) cells to recognize and eliminate "missing self" target cells *in vivo*—a process involving complex balancing interactions between activating and inhibitory NK cell receptors. Such screens are not just limited to identifying the presence/absence of NK cells and/or $CD8^+$ T cells *in vivo* but challenges the host response to undergo NK cell recognition/killing, antigen uptake/ processing and presentation by Dendritic Cells (DCs), ultimately causing T cell priming, expansion and T cell cytolytic effector function. The *in vivo* immune responses assess the capacity of ENU mice to induce sterile inflammatory responses mediated by self-molecules that activate either NK cells and/or Toll-like receptor-independent sensing pathways—the latter presumably activated by cell-death- or "danger-" associated molecular patterns (DAMPs). Importantly, the induction of type I or II IFNs are essential for the generation of antigen-specific T cell responses mediated via cell-death induced immune responses[8]. Whereas IFNs have been shown to promote the maturation of DCs and stimulate T cell priming, the underlying pathways inducing type I IFN following exposure of DCs to dying cells are less well defined. It is well established that host molecules such as DNA and/or RNA in apoptotic cells can cause sustained and systemic type I IFN production when they escape degradation in macrophages[9-11]. The pathways by which such nucleotide structures drive type I IFN production following administration of apoptotic cells remains still elusive to date. Thus, the *in vivo* cytotoxicity screen performed in our laboratory presents a large genetic footprint, not only comprising lymphocyte development but also targeting NK-, DC- and T cell biological function. As a result, we have identified a number of germline mutants that are either deficient in the IFN pathways, but also includes germline mutants that exhibit impaired lymphocyte survival, T cell activation and/or actin-polymerization. Here we will provide two examples how ENU germline mutants can provide new insight into gene function, immunological pathways and/or disease development.

4. Gimap5 and loss of immunological tolerance driving auto-immune diseases

Using N-ethyl-N-nitrosourea (ENU) germline mutagenesis, our laboratory previously identified Gimap5-deficient mice—designated *sphinx*—that exhibit reduced lymphocyte

survival and develop severe colitis around 10-12 weeks of age[12]. Specifically, these mice lack NK or $CD8^+$ T cell populations in peripheral lymphoid organs, whereas relatively normal thymocyte development occurs, including the $CD4^+$ T cell, $CD8^+$ T cell, and $Foxp3^+$ regulatory T cell lineages. Coarse mapping and sequencing of the critical region revealed a single G→T point mutation in *Gimap5* to be the causal mutation. This mutation resulted in a G38C amino acid substitution in the predicted GTP-binding domain of Gimap5, destabilizing Gimap5 protein expression[12].

Gimap5 is part of the family of Gimap genes which are predominantly expressed in lymphocytes and regulate lymphocyte survival during development and homeostasis[13]. Gimap proteins contain a GTP-binding AIG1 homology domain, first identified in disease-resistance genes in higher plants[9, 10]. More recent crystallographic studies showed that the Gimap proteins resemble a nucleotide coordination and dimerization mode previously observed for dynamin GTPase—a component essential for the scission and fusion of cellular vesicular compartments such as endosomes at the cell surface or the Golgi apparatus in the cytosol[14]. Members of the Gimap family appear to be localized to different subcellular compartments with Gimap5 reported to localize in lysosomes based on studies in human, mouse and rat lymphocytes[15]. Overall, the function of these proteins and their role in disease development remain poorly defined.

Genetic aberrancies in Gimap5 have been strongly linked to reduced lymphocyte survival and homeostasis, but importantly have also been associated with autoimmune diseases. In humans, polyadenylation polymorphisms in GIMAP5—causing relative modest changes in GIMAP5 RNA expression—were associated with increased concentrations of IA2 auto-antibodies in type 1 diabetes (T1D) patients and an increased risk of systemic lupus erythematosus (SLE)[16, 17]. Studies using biobreeding (BB) rats— carrying a mutation (*lyp/lyp*) in Gimap5— show marked lymphopenia and predisposition to the development of T1D[18-22] and intestinal inflammation[23]. Together these observations suggest that, beyond lymphocyte survival, Gimap5 is essential for maintaining immunological tolerance.

Although in $Gimap5^{sph/sph}$ mice no auto-antibodies can be detected, males and females developed severe colitis around 8-12 weeks of age, which was dependent on the microbiome and is $CD4^+$ T cell driven[12]. Interestingly, inflammatory bowel disease (IBD) such as Crohn's disease, ulcerative colitis and indeterminate colitis[24, 25] manifest generally in adolescence or adulthood and they behave as complex, polygenic diseases often sharing common risk factors with other autoimmune diseases[26, 27]. Previous work suggests that impaired lymphocyte survival and consequent lymphopenia may be linked to the loss of immunological tolerance. Specifically, $CD4^+$ T cells in a lymphopenic environment can undergo thymic independent expansion in the periphery. This process—also referred to as lymphopenia-induced proliferation (LIP)—is accompanied by marked alterations in T cell phenotype and is linked to auto-immunity[28-30]. Most notably, $CD4^+$ T cells more readily adopt an effector phenotype, including the ability to robustly produce cytokines and can drive the development of colitis[31-33]. Importantly, the absence of Treg cells is an important determinant of immune-mediated sequelae, including colitis that is induced by $CD4^+$ T cells

undergoing LIP. Interestingly, studies in our laboratory show that in *Gimap5*$^{sph/sph}$ mice, the onset of colitis is preceded by a progressive reduction in circulating CD4$^+$ T cells with remaining CD4$^+$ T cells exhibiting a lymphopenia-induced proliferation (LIP) phenotype (CD44^{high} and CD62^{low}) with a large number of cells in S phase([12] and Figure 1). Moreover, CD4$^+$ T cells derived from *Gimap5*$^{sph/sph}$ spleen or mesenteric lymph nodes (MLNs) exhibit a higher capacity to produce cytokines, i.e. IFNγ and/or IL-17A following activation of the T cell receptor.

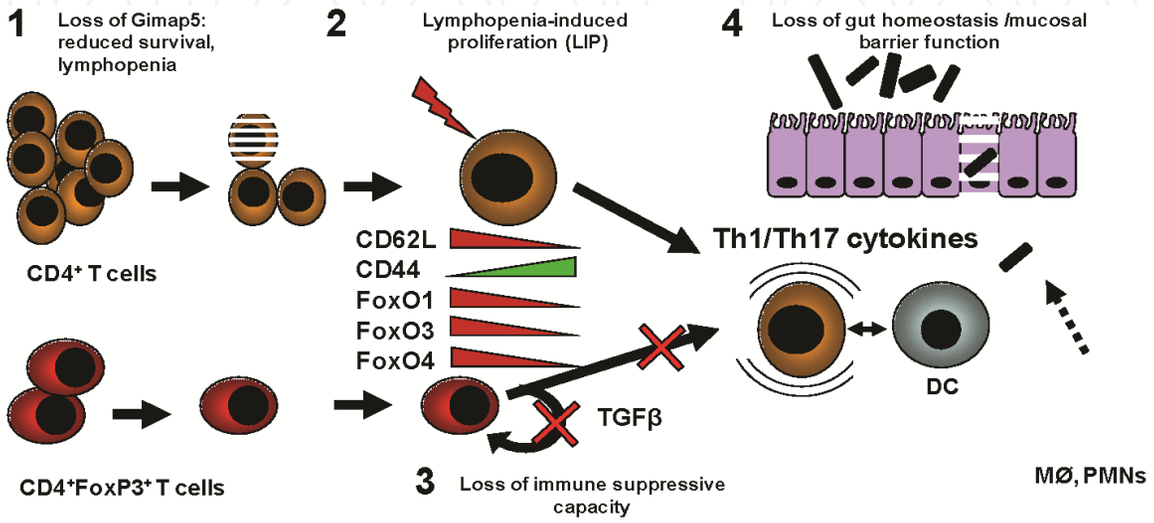

Figure 1. Schematic representation of the events causing colitis in Gimap5-deficient mice. Loss of Gimap5 leads to reduced survival of lymphocytes including CD4$^+$ T cells (1). During lymphopenia, CD4$^+$ T cells undergo LIP exemplified by increased surface expression of CD44 and reduced levels of CD62L (2). Concomitantly, CD4$^+$ T cells exhibit loss of full-length FoxO1, FoxO3 and FoxO4 expression, affecting both immunosuppressive function or Treg cells and the induction of Treg cells in the mesenteric lymph nodes (3). Together these events promote Th17 differentiation and activation of CD4$^+$ T cells in the gut causing inflammation and infiltration of macrophages / neutrophils that further amplify intestinal inflammation (4).

Given the important role of regulatory T cells in immune-mediated sequelae induced by CD4$^+$ T cells undergoing LIP, our laboratory assessed whether the colitis was driven by abnormalities in regulatory T cell development or function. Although relatively normal numbers of Foxp3$^+$ Treg cells are found in 3-week-old mice, a loss of Treg cell numbers is observed by 6 weeks of age particularly in the MLNs[34]. In addition, regulatory T cells in *Gimap5*$^{sph/sph}$ mice show a progressive loss of suppressive function. Specifically, whereas Treg cells from 4-week-old *Gimap5*$^{sph/sph}$ mice show a slight, but significant reduction in their ability to suppress CD8$^+$ T cell proliferation *in vitro*, Treg cells isolated from 6-week-old *Gimap5*$^{sph/sph}$ mice are incapable of suppressing CD8$^+$ T cell proliferation entirely, thus indicating that a progressive impairment in Treg cell survival and function may underlie the colitis development in *Gimap5*$^{sph/sph}$ mice. Indeed, colitis can be prevented entirely by injecting wildtype regulatory T cells in 4-week-old *Gimap5*$^{sph/sph}$ mice.

Interestingly, the T cell phenotypes in *Gimap5*$^{sph/sph}$ mice show striking similarities with those seen in mice deficient in the family of Forkheadbox group O (Foxo) transcription factors.

The family of Foxo transcription factors contain 4 members of which three (Foxo1, Foxo3 and Foxo4) have overlapping patterns of expression and transcriptional activities[35-37]. They play an essential role in the quiescence and survival of $CD4^+$ T cells. Foxo1 expression is critical for maintaining naïve T cell quiescence[38-40]. In addition, Foxo1, 3 expression has been reported to be essential for Treg cell development and function[41, 42]. Specifically, Foxo transcription factors serve a role as coactivators downstream of the TGFβ signaling pathway by interacting with SMAD proteins[43, 44], and directly regulate the induction of a number of Treg cell associated genes, including Foxp3, CTLA-4 and CD25[41, 42]. Indeed immunoblot analysis of $CD4^+$ T (including Treg cells) from *Gimap5*$^{sph/sph}$ mice at various ages, revealed a progressive loss of full-length Foxo1, -3a and -4 proteins, with normal levels at 3 weeks of age, but a complete loss of Foxo-expression in $CD4^+$ T cells from 6-10 week-old *Gimap5*$^{sph/sph}$ mice[34]. The regulation of Foxo3 and Foxo4 protein expression appears to occur at the post-transcriptional level, although the exact mechanism underlying the loss of Foxo-expression remains to be determined. The progressive nature suggest a strong association with the loss of Treg function in *Gimap5*$^{sph/sph}$ over time and link the loss of full-length Foxo expression in *Gimap5*$^{sph/sph}$ lymphocytes with the onset of lymphopenia, impaired lymphocyte proliferation and increased effector function and differentiation into Th17 cells (Figure 1). The detailed mechanistic insight into the loss of immunological tolerance occurring in *Gimap5*$^{sph/sph}$ mice may ultimately provide important leads as to how polyadenylation polymorphisms in GIMAP5 predispose human patients to T1D or SLE.

5. Mutations in hematopietic protein 1; an immunodeficiency resulting in loss of a broad range of immunological functions

Genetic aberrancies causing severe combined immunodeficiency (SCID) are generally rare and associated with a high morbidity and/or mortality. They often present significant challenges in terms of treatment due to the wide variety of immune cells that can be affected. Therefore, besides defining the genetic footprint underlying SCID, a critical challenge lies in obtaining a thorough understanding of the degree of the immunodeficiency presented by specific mutations in genes, including defining the types of immune cells affected and functional aberrancies observed. Our laboratory previously identified a germline mutant, designated *Lampe2*, which exhibited impaired NK as well as $CD8^+$ T cell cytolytic effector function as determined by the *in vivo* cytotoxicity assay described above (Figure 2a). The G1 pedigrees of these germline mutants were selected to establish a homozygote colony used for genetic analysis and further phenotypic characterization. The mutation behaved as strictly recessive, in that normal cytolytic effector functions were observed in heterozygote mutant mice. Further characterization of 6-week-old homozygote *Lampe2* mutants revealed markedly reduced numbers of $CD8^+$ T, $CD4^+$ T and B cell populations, and a slight reduction in NK cells (Figure 2b). In contrast, an increase in the number of macrophages in the spleen was observed (Figure 2b). Notably, upon necropsy, the liver exhibited white patches at the periphery (Figure 2c) which upon histological analysis revealed large areas of necrosis and significant hematopoietic infiltrate and inflammation (Figure 2d-e).

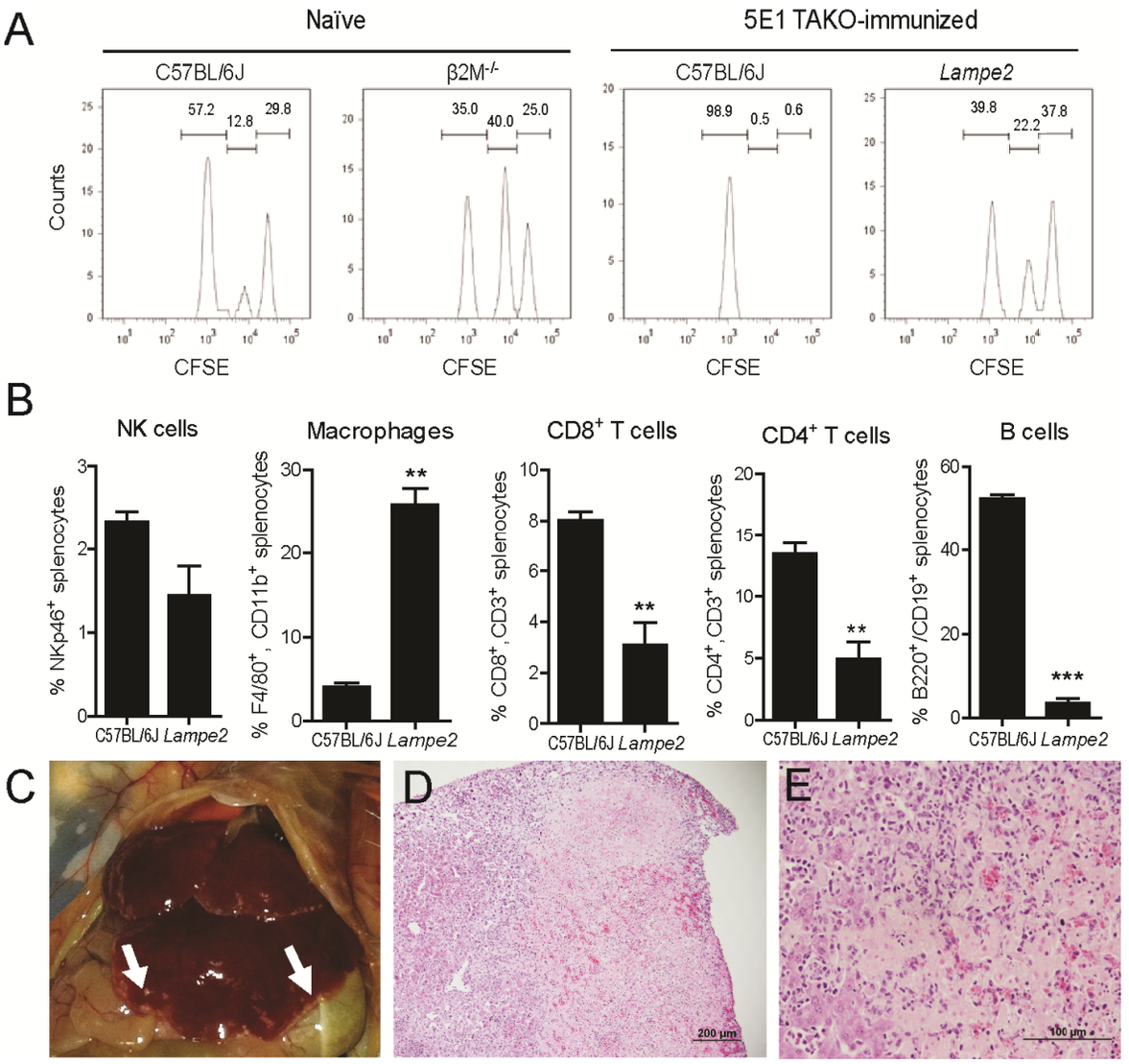

Figure 2. Impaired NK and CD8⁺ T cell function and development of liver injury in *Lampe2* mice. (**a**) Reduced clearance of CFSE labeled β-2m-deficient and antigen-specific target splenocytes in *Lampe 2* germline mutants compared to C57BL/6J control mice *in vivo*. 48 hours after transfer, blood samples were collected and analyzed for the presence of wildtype splenocytes (low-CFSE) and Kb-deficient splenocytes (medium-CFSE). The percentage killing is calculated from the ratio between β-2m-deficient and C57BL/6J cells administered to β2m-deficient and control naïve C57BL/6J recipients. (**b**) The percentage of NK cells, macrophages, CD8⁺ T cells, CD4⁺ T cells and B cells in C57BL/6J and homozygote *Lampe2* mutant mice. (n > 3) (**c**) Macroscopic and histological analysis of *Lampe2* livers.*= P<0.05; **= P<0.01; ***= P<0.001

To identify the causative mutation in *Lampe2* mice, we performed coarse mapping by crossing *Lampe2* C57BL/6J homozygotes males with 129S1/SvImJ females. The resulting F1 offspring were intercrossed to generate a F2 and a total of 24 offspring (6 *Lampe2* mutant- and 18 wildtype-phenotypes) were analyzed for both phenotype and genotype. Genotyping was performed using a genome wide custom-made 353-SNP map distinguishing C57BL/6J and 129S1/SvImJ genetic backgrounds. Coarse mapping revealed a single peak with a LOD score of ~5.86 for SNP rs13482738 located on the distal end of chromosome 15 (Figure 3A).

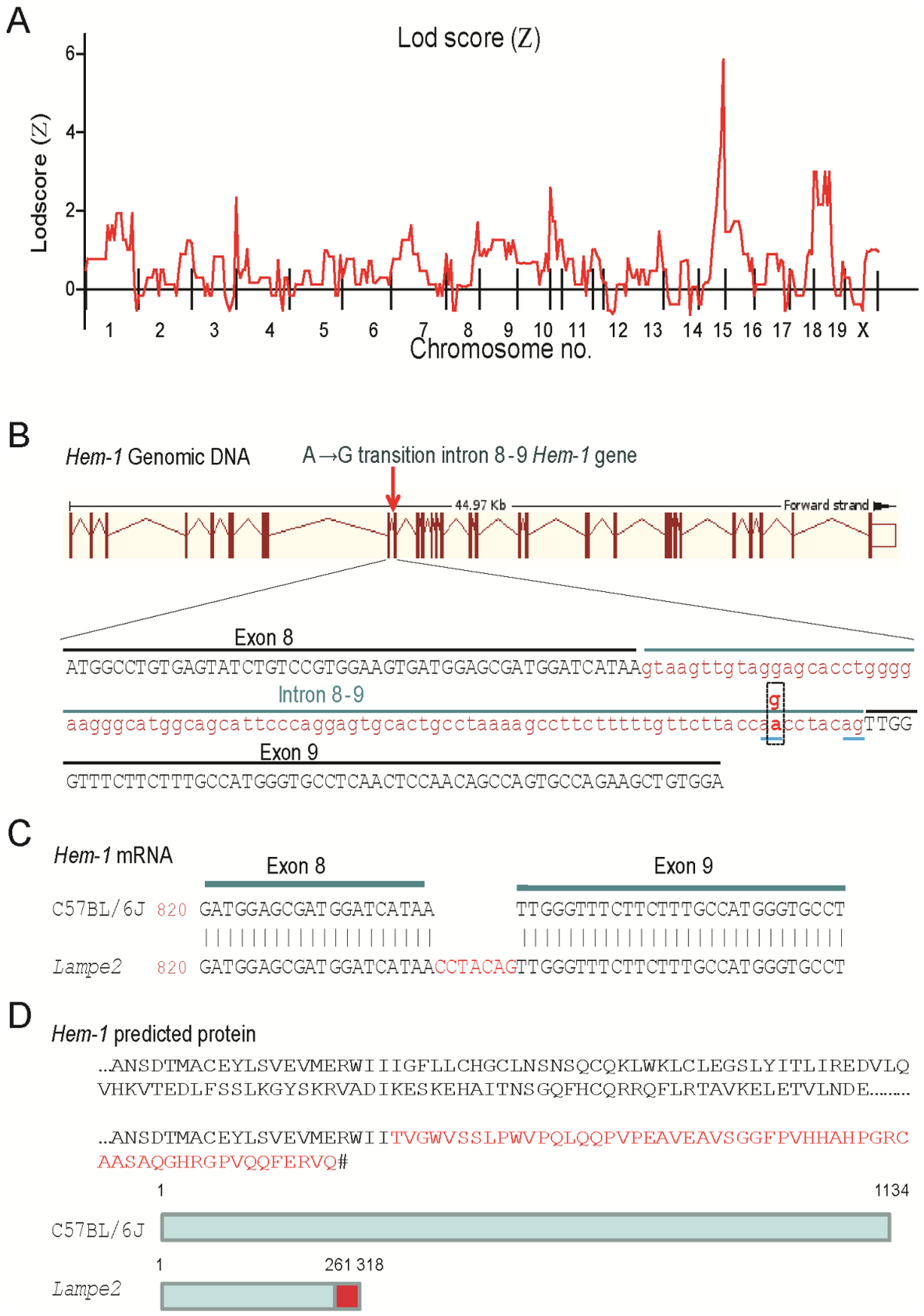

Figure 3. Coarse mapping and identification of the causative mutation in *Lampe2* mice. (a) Low-resolution mapping of the *Lampe2* mutation based on twenty-four mice and a panel of 353 SNPs

covering the entire genome. The *Lampe2* phenotype was linked to the distal site of chromosome 15. (**b,c**), the A→G intronic mutation causes a new acceptor splice site (**b**) resulting in the inclusion of 7 nucleotides intronic sequence into mature *Hem1* transcript as determined by sequencing of *Hem1* cDNA (**c**). (**d**) At the protein level, the mutation is predicted to cause a frameshift at amino acid 261 with alternative coding and premature stop at amino acid 318, resulting in a largely truncated Hem1 protein.

The critical region was defined by proximal marker rs6285067 (at position 95.14 Mb) and the distal end of chromosome 15 (at 103.40 Mb) and consisted of ~8.26 Mb genomic DNA containing 242 annotated genes. Among the annotated genes, *Hematopoietic protein 1* (*Hem-1* aka *NCK associated protein 1 like* or *Nckap1l*) presented a clear candidate gene, in that a previously reported ENU germline mutant carrying a point mutation (referred to as the NBT.1 mutation) causing a premature stop and absence of protein expression, exhibits striking similar phenotypes compared to *Lampe2* mice. Specifically, these mice exhibit lymphopenia with a reduced number of peripheral $CD4^+$ T, $CD8^+$ T and B cells, and on the other hand showed marked expansion of myeloid cells, including neutrophils and macrophages. Moreover, the liver phenotype in mice carrying the NBT.1 mutation bears high resemblance with the liver phenotype observed in *Lampe2* mice—i.e. the occurrence of whitish liver margins and large areas of inflammation. *Hem1* is a member of the Hem family of cytoplasmic adaptor molecules predominantly and is expressed exclusively in hematopoietic cells, including T and B cells, macrophages, DCs and granulocytes. Hem1 plays a critical role in the reorganization of actin cytoskeleton and as such, affects a wide variety of immune functions, including chemotaxis/migration, adhesion, formation of an immune synapse and phagocytosis. Sequencing of *Hem1* exons, including 50 bps of proximal/distal intronic sequence, was performed by Sanger sequencing methodology using genomic DNA and revealed a single A→G point mutation in intron 8-9 located 6 nucleotides upstream of the exon 9 acceptor splice site (Figure 3b). The A→G intronic nucleotide change potentially presented a new acceptor splice site and indeed sequencing of *Hem-1* mRNA isolated from spleen showed the inclusion of 7 intronic nucleotides in mRNA derived from *Lampe2* mice (Figure 3c). At the protein level, the inclusion of intronic nucleotides is predicted to result in a frame-shift and alternative coding following residue 261, and a premature stop at amino acid 381 resulting in a largely truncated Hem-1 protein in *Lampe2* mice (Figure 3d). Given the similarities of the *Lampe2* and *NBT.1* mutant phenotypes and the predicted severe impact of the *Lampe2* mutation on Hem-1 protein expression, we concluded that the mutation in Hem-1 caused the observed phenotypes in *Lampe2* mice (hereafter referred to as $Hem1^{lampe2}$).

Hem1 is part of the Wiskott-Aldrich syndrome protein family Verprolin-homologous protein (WAVE) protein complex in hematopoietic cells regulating cell mobility and intracellular processes requiring rearrangement of the cytoskeleton following immuno-receptor activation, including B and T cell, chemokine and innate immune receptors such as Toll-like receptors. Specifically, receptor triggering causes activation of Rho family of Guanosine triphosphatases (GTPases) such as CDC42, RhoA and Rac ultimately resulting in the activation of downstream adaptor complexes involved in the regulating of actin (de)polymerization. For hematopoietic cells, the adaptor complexes Wiskott-Aldrich

syndrome protein (WASP) and WAVE are particularly important for the control of actin polymerization[45-48]. The hematopoietic cell-specific WAVE complex consists of a pentameric subunit complex including, Sra-1 (Specifically Rac-associated protein-1), Hem1, Abi (Abelson interactor 1 or 2), WAVE, and HSPC300 (Hematopoietic stem/progenitor cell protein 300)[49]. Under non-stimulated conditions, the WAVE complex is inactive, but following immunoreceptor activation, GTP-bound Rac binds the pentameric complex presumably through Sra1[49]. In addition, this complex requires binding of phophatidylinositol (3,4,5) triphosphate (PIP_3) interaction and phosphorylation by kinases[50], including Abl kinase and Mitogen-activated protein kinases[51]. Ultimately, this results in a conformational change revealing the WAVE-specific VCA (Verprolin-homology, Cofillin-homology, and acidic) region and allow interaction with the actin-regulatory complex (Arp2/3), ultimately converting monomeric actin (G-actin) into filamentous actin (F-actin). Interestingly, the absence of individual subunit components often causes the degradation of all components of the WAVE complex resulting in aberrant actin polymerization. The consequences of deregulated actin polymerization in hematopoietic cells are wide-ranging and affect broad immunological functions, including but not limited to: 1) leukocyte migration/chemotaxis, 2) loss of immune synapse formation affecting T and B cell receptor signaling (thereby affecting T cell function and development), 3) leukocyte adhesion, and 4) DC-specific phagocytosis and their ability to cross present/prime T cells. As such, mutations in the specific subunit components of the WAVE complex resulting in abnormal gene expression/function cause severe combined immunodeficiencies that stretch beyond lymphocyte populations also affecting granulocyte function and are predicted to correlate with high mortality/morbidity.

6. Implications for human PID

Assessing the immune system using ENU mutagenesis in mice has previously led to important breakthrough discoveries in understanding the genetics in human patients with PID. A prime example is the identification of the *3d* allele—a missense allele of *Unc93b1*, a gene encoding an ER membrane protein with 12 membrane spanning motifs with a previously unknown function. *3d* germline mutants were identified in a screen probing the response of macrophages derived from ENU germline mice to a variety of TLR-ligands. Homozygote *3d* mutant mice were found to be unresponsive to ligands activating endosomal TLRs, but exhibited normal responses to TLRs expressed at the surface[39]. Interestingly, at the same time Casrouge et al. identified two unrelated human patients that presented recurrent infections with Herpes simplex virus-1 (HSV-1) resulting in encephalitis (HSE) and showed remarkable similarities between the phenotypes observed in the human patients and in *3d* mutant mice. Specifically, both patients were unresponsive to endosomal TLR stimulation and showed a high viral susceptibility. Following the identification of the causative mutation in 3d mice as being a missense allele of *Unc93b1*, subsequent sequencing of the human patients indeed revealed aberrant mutations in *UNC93B*[52]. This example highlights the power of ENU mutagenesis and its

unbiased approach, by uncovering the function of genes for which a biological function is otherwise difficult to predict.

With regard to the *Gimap5* and *Hem-1* germline mutations described in this chapter, both present examples of genetic mutations leading to severe combined immunodeficiencies. Although limited information is available with regard to genetic mutations causing a null phenotype in human *GIMAP5* or *HEM1,* ample evidence exist that dysregulation of these genes plays an important role in human disease. A previous report suggests that SLE patients were shown to have a trend for lower GIMAP5 mRNA expression in peripheral blood mononuclear cells compared to healthy controls[16]. Moreover, a poly-adenylation mutation in the 3′ region of *GIMAP5*, resulting in minor changes in *GIMAP5* mRNA expression in peripheral blood mononuclear cells, are associated with increased predisposition to SLE and T1D[15, 16, 53]. Thus far, the effect on GIMAP protein expression, specifically in lymphocytes of homozygote/heterozygote carriers for this mutation, remains elusive and warrants further research. Our studies using *Gimap5*-deficient mice point to an important role for Gimap5 in maintaining peripheral immunological tolerance that is intrinsically related to the loss of Gimap5 expression in $CD4^+$ T cells. Thus, research efforts may be directed to a better understanding of GIMAP5 and FOXO protein expression specifically in $CD4^+$ T cells in human patients with SLE or T1D that carry the polyadenylation mutation in GIMAP5.

Finally, perhaps due to its indispensable role in a wide variety of immune pathways, mutations in human HEM1 leading to dysregulated actin polymerization, have thus far not been reported. Nonetheless, over- or under-expression of HEM1 is associated with disease prognosis in leukemia[54]. Specifically, HEM1 overexpression in B-cell chronic lymphocytic leukemia (CLL) is associated with a poor outcome, whereas down-regulation of HEM1 expression in CLL cells rendered tumor cells more susceptible to fludarabine-mediated killing[54]. These findings may indicate the critical role for HEM1 in invasion and/or metastasis of tumor cells from hematopoietic origin.

7. Concluding remarks

A major challenge in the field of genomics is to obtain a comprehensive understanding of the functions of all annotated mammalian genes. Whereas identification and analysis of genome wide SNPs and/or unique nucleotide changes are drastically improved following the development of next generation sequencing technologies, understanding the consequences of such genetic variants remains a major challenge in virtually all biomedical fields. ENU mutagenesis provides one approach that is both powerful and unbiased, uncovering gene function by introducing the sort of genetic abnormalities that can be observed in human patients (e.g. primary immuno-deficiencies). Ultimately, utilization of both forward and reverse genetic approaches will be instrumental in closing the existing phenotype gap and will help us understand the association between identified genetic variants, the implications for protein and biological function, and human disease.

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8. References

[1] Visscher PM, Brown MA, McCarthy MI, Yang J. Five years of GWAS discovery. Am J Hum Genet 2012; 90:7-24.
[2] Bousfiha A, Picard C, Boisson-Dupuis S, Zhang SY, Bustamante J, Puel A, et al. Primary immunodeficiencies of protective immunity to primary infections. Clin Immunol 2010; 135:204-9.
[3] Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; 282:2085-8.
[4] Brown SD, Peters J. Combining mutagenesis and genomics in the mouse--closing the phenotype gap. Trends Genet 1996; 12:433-5.
[5] Sheridan R, Lampe K, Shanmukhappa SK, Putnam P, Keddache M, Divanovic S, et al. Lampe1: an ENU-germline mutation causing spontaneous hepatosteatosis identified through targeted exon-enrichment and next-generation sequencing. PLoS One 2011; 6:e21979.
[6] Justice MJ, Noveroske JK, Weber JS, Zheng B, Bradley A. Mouse ENU mutagenesis. HumMolGenet 1999; 8:1955-63.
[7] Beutler B, Du X, Xia Y. Precis on forward genetics in mice. NatImmunol 2007; 8:659-64.
[8] Krebs P, Barnes MJ, Lampe K, Whitley K, Bahjat KS, Beutler B, et al. NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses. Blood 2009; 113:6593-602.
[9] Kawane K, Ohtani M, Miwa K, Kizawa T, Kanbara Y, Yoshioka Y, et al. Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. Nature 2006; 443:998-1002.
[10] Okabe Y, Kawane K, Akira S, Taniguchi T, Nagata S. Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. JExpMed 2005; 202:1333-9.

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[11] Tsukumo S, Yasutomo K. DNaseI in pathogenesis of systemic lupus erythematosus. ClinImmunol 2004; 113:14-8.
[12] Barnes MJ, Aksoylar H, Krebs P, Bourdeau T, Arnold CN, Xia Y, et al. Loss of T cell and B cell quiescence precedes the onset of microbial flora-dependent wasting disease and intestinal inflammation in Gimap5-deficient mice. J Immunol 2010; 184:3743-54.
[13] Nitta T, Nasreen M, Seike T, Goji A, Ohigashi I, Miyazaki T, et al. IAN family critically regulates survival and development of T lymphocytes. PLoSBiol 2006; 4:e103.
[14] Schwefel D, Frohlich C, Eichhorst J, Wiesner B, Behlke J, Aravind L, et al. Structural basis of oligomerization in septin-like GTPase of immunity-associated protein 2 (GIMAP2). Proc Natl Acad Sci U S A 2010; 107:20299-304.
[15] Wong V, Saunders A, Hutchings A, Pascall J, Carter C, Bright N, et al. The auto-immunity-related GIMAP5 GTPase is a lysosome-associated protein. self/Nonself 2010; 1:9.
[16] Hellquist A, Zucchelli M, Kivinen K, Saarialho-Kere U, Koskenmies S, Widen E, et al. The human GIMAP5 gene has a common polyadenylation polymorphism increasing risk to systemic lupus erythematosus. J Med Genet 2007; 44:314-21.
[17] Shin JH, Janer M, McNeney B, Blay S, Deutsch K, Sanjeevi CB, et al. IA-2 autoantibodies in incident type I diabetes patients are associated with a polyadenylation signal polymorphism in GIMAP5. Genes Immun 2007; 8:503-12.
[18] Hornum L, Romer J, Markholst H. The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. Diabetes 2002; 51:1972-9.
[19] Jacob HJ, Pettersson A, Wilson D, Mao Y, Lernmark A, Lander ES. Genetic dissection of autoimmune type I diabetes in the BB rat. NatGenet 1992; 2:56-60.
[20] Macmurray AJ, Moralejo DH, Kwitek AE, Rutledge EA, Van Yserloo B, Gohlke P, et al. Lymphopenia in the BB rat model of type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. Genome Res 2002; 12:1029-39.
[21] Ramanathan S, Poussier P. BB rat lyp mutation and Type 1 diabetes. ImmunolRev 2001; 184:161-71.
[22] van den Brandt J, Fischer HJ, Walter L, Hunig T, Kloting I, Reichardt HM. Type 1 diabetes in BioBreeding rats is critically linked to an imbalance between Th17 and regulatory T cells and an altered TCR repertoire. J Immunol 2010; 185:2285-94.
[23] Cousins L, Graham M, Tooze R, Carter C, Miller JR, Powrie FM, et al. Eosinophilic bowel disease controlled by the BB rat-derived lymphopenia/Gimap5 gene. Gastroenterology 2006; 131:1475-85.
[24] Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002; 347:417-29.
[25] Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007; 448:427-34.
[26] Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. Gut 2011.
[27] Mackay IR. Clustering and commonalities among autoimmune diseases. J Autoimmun 2009; 33:170-7.
[28] Khoruts A, Fraser JM. A causal link between lymphopenia and autoimmunity. Immunol Lett 2005; 98:23-31.

[29] King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004; 117:265-77.
[30] Krupica T, Jr., Fry TJ, Mackall CL. Autoimmunity during lymphopenia: a two-hit model. Clin Immunol 2006; 120:121-8.
[31] Aranda R, Sydora BC, McAllister PL, Binder SW, Yang HY, Targan SR, et al. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. J Immunol 1997; 158:3464-73.
[32] Powrie F, Correa-Oliveira R, Mauze S, Coffman RL. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. J Exp Med 1994; 179:589-600.
[33] Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol 1993; 5:1461-71.
[34] Aksoylar HI, Lampe K, Barnes MJ, Plas DR, Hoebe K. Loss of Immunological Tolerance in Gimap5-Deficient Mice Is Associated with Loss of Foxo in CD4+ T Cells. J Immunol 2012; 188:146-54.
[35] Anderson MJ, Viars CS, Czekay S, Cavenee WK, Arden KC. Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. Genomics 1998; 47:187-99.
[36] Biggs WH, 3rd, Cavenee WK, Arden KC. Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse. Mamm Genome 2001; 12:416-25.
[37] Furuyama T, Kitayama K, Shimoda Y, Ogawa M, Sone K, Yoshida-Araki K, et al. Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. J Biol Chem 2004; 279:34741-9.
[38] Kerdiles YM, Beisner DR, Tinoco R, Dejean AS, Castrillon DH, DePinho RA, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. NatImmunol 2009; 10:176-84.
[39] Ouyang W, Beckett O, Flavell RA, Li MO. An essential role of the Forkhead-box transcription factor Foxo1 in control of T cell homeostasis and tolerance. Immunity 2009; 30:358-71.
[40] Tothova Z, Gilliland DG. FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system. Cell Stem Cell 2007; 1:140-52.
[41] Kerdiles YM, Stone EL, Beisner DL, McGargill MA, Ch'en IL, Stockmann C, et al. Foxo transcription factors control regulatory T cell development and function. Immunity 2010; 33:890-904.
[42] Ouyang W, Beckett O, Ma Q, Paik JH, DePinho RA, Li MO. Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nat Immunol 2010; 11:618-27.
[43] Gomis RR, Alarcon C, He W, Wang Q, Seoane J, Lash A, et al. A FoxO-Smad synexpression group in human keratinocytes. Proc Natl Acad Sci U S A 2006; 103:12747-52.
[44] Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell 2004; 117:211-23.

[45] Park H, Chan MM, Iritani BM. Hem-1: putting the "WAVE" into actin polymerization during an immune response. FEBS Lett 2010; 584:4923-32.
[46] Park H, Staehling-Hampton K, Appleby MW, Brunkow ME, Habib T, Zhang Y, et al. A point mutation in the murine Hem1 gene reveals an essential role for Hematopoietic protein 1 in lymphopoiesis and innate immunity. J Exp Med 2008; 205:2899-913.
[47] Thrasher AJ, Burns SO. WASP: a key immunological multitasker. Nat Rev Immunol 2010; 10:182-92.
[48] Williams DA, Tao W, Yang F, Kim C, Gu Y, Mansfield P, et al. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. Blood 2000; 96:1646-54.
[49] Gautreau A, Ho HY, Li J, Steen H, Gygi SP, Kirschner MW. Purification and architecture of the ubiquitous Wave complex. Proc Natl Acad Sci U S A 2004; 101:4379-83.
[50] Lebensohn AM, Kirschner MW. Activation of the WAVE complex by coincident signals controls actin assembly. Mol Cell 2009; 36:512-24.
[51] Danson CM, Pocha SM, Bloomberg GB, Cory GO. Phosphorylation of WAVE2 by MAP kinases regulates persistent cell migration and polarity. J Cell Sci 2007; 120:4144-54.
[52] Casrouge A, Zhang SY, Eidenschenk C, Jouanguy E, Puel A, Yang K, et al. Herpes simplex virus encephalitis in human UNC-93B deficiency. Science 2006; 314:308-12.
[53] Lim MK, Sheen DH, Kim SA, Won SK, Lee SS, Chae SC, et al. IAN5 polymorphisms are associated with systemic lupus erythematosus. Lupus 2009; 18:1045-52.
[54] Joshi AD, Hegde GV, Dickinson JD, Mittal AK, Lynch JC, Eudy JD, et al. ATM, CTLA4, MNDA, and HEM1 in high versus low CD38 expressing B-cell chronic lymphocytic leukemia. Clin Cancer Res 2007; 13:5295-304.