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Timothy E. Dudek *et al.*

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Editor's Summary

Mirror, Mirror

One limitation of using animal models of disease is that there's no magic mirror to tell you which one best reflects human disease. Instead, most animal disease models mimic some aspects of the human condition, but may not recapitulate the disease in its entirety. This limitation is especially true for HIV infection because the virus does not naturally infect mice —the model of choice for biomedical research. Attempts to "humanize" immunodeficient mice through grafting of human immune cells may reconfigure the mouse from a distorting funhouse mirror into a well-lit vanity one. Now, Dudek *et al*. use humanized BLT (brain, liver, thymus) mice to study human immune responses to HIV.

The authors found that HIV-1–specific immune responses in BLT mice mimicked those in humans in terms of specificity, kinetics, and dominant target. Importantly, HIV adapted to the immune responses in these mice just as it does in humans, evolving rapidly to escape from the selective pressure. Indeed, an HLA allele that is protective in humans induced similar protective immune responses in these mice. Although no animal model may perfectly reflect human disease, for HIV infection, humanized BLT mice may be one of the fairest of them all.

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HIV

Rapid Evolution of HIV-1 to Functional CD8$^+$ T Cell Responses in Humanized BLT Mice

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The development of mouse/human chimeras through the engraftment of human immune cells and tissues into immunodeficient mice, including the recently described humanized BLT (bone marrow, liver, thymus) mouse model, holds great promise to facilitate the in vivo study of human immune responses. However, little data exist regarding the extent to which cellular immune responses in humanized mice accurately reflect those seen in humans. We infected humanized BLT mice with HIV-1 as a model pathogen and characterized HIV-1–specific immune responses and viral evolution during the acute phase of infection. HIV-1–specific CD8$^+$ T cell responses in these mice were found to closely resemble those in humans in terms of their specificity, kinetics, and immunodominance. Viral sequence evolution also revealed rapid and highly reproducible escape from these responses, mirroring the adaptations to host immune pressures observed during natural HIV-1 infection. Moreover, mice expressing the protective HLA-B*57 allele exhibited enhanced control of viral replication and restricted the same CD8$^+$ T cell responses to conserved regions of HIV-1 Gag that are critical to its control of HIV-1 in humans. These data reveal that the humanized BLT mouse model appears to accurately recapitulate human pathogen–specific cellular immunity and the fundamental immunological mechanisms required to control a model human pathogen, aspects critical to the use of a small-animal model for human pathogens.

INTRODUCTION

Animal models are critical to our understanding of human pathogens and diseases. The capacity to monitor, induce, and augment immune responses in animal models has been integral to the development of therapeutics and vaccines. However, many human pathogens do not successfully infect typical laboratory animal species, requiring the extrapolation of data acquired from models of pathogens similar, but not identical, to those infecting humans. In the case of HIV-1, the model of choice is the simian immunodeficiency virus (SIV)–infected rhesus macaque, which has proved invaluable for studying the mechanisms of disease pathogenesis and vaccine efficacy (*1*, *2*). Nonetheless, differences in host genetics between humans and macaques, particularly in their major histocompatibility complex class I (MHC-I) alleles and T cell receptor repertoire, as well as substantial sequence differences between HIV-1 and SIV, prevent the study of HIV-1–specific human immune responses in macaques. Additionally, although studies of genetically similar macaques or identical subjects can facilitate a more stringent interpretation of data (*3*, *4*), the inherent genetic diversity among macaques typically complicates the interpretation of experimental results. Thus, improved animal models that are capable of more accurately modeling natural human immune responses to HIV-1 infection and exhibit a high degree of genetic homogeneity could substantially accelerate biomedical research and vaccine development for HIV-1 as well as a number of other human pathogens (*5*, *6*).

The recent generation of humanized BLT (bone marrow, liver, thymus) mice, through the implantation of human fetal thymic and liver tissues along with the transfer of human fetal liver–derived CD34$^+$ hematopoietic stem cells (HSCs), has markedly improved the ability of mice to support human immune system reconstitution. In these BLT mice, human T cells may become educated within autologous human thymic tissues, rather than within xenogenic mouse tissues, resulting in a significantly more robust repopulation of human immune cells. Although these and other humanized mice have been shown to develop cellular immune responses against pathogens and/or immunogens (*7*–*12*), little evidence exists on the specificity or the functionality of these responses or on the ability of these responses to control infections. Therefore, the extent to which cellular immune responses in these animals accurately reflect those seen during natural human infection and exhibit control of pathogens remains unclear.

Although humanized BLT mice support sustained levels of HIV-1 replication (*8*, *13*, *14*), reports describing the development of HIV-1–specific cellular immune responses have been inconsistent, with some groups detecting responses (*8*, *11*), whereas others note a paucity of detectable responses (*13*, *15*). Here, we detected and characterized CD8$^+$ T cell responses and early viral sequence evolution against this model pathogen. Our results indicate that the kinetics, magnitude, specificity, and immunodominance hierarchies of human HIV-1–specific CD8$^+$ T cell responses mounted in humanized BLT mice during the acute phase of infection appear to closely resemble those of HIV-1–infected humans.

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RESULTS

Human immune system reconstitution in BLT mice allows for productive infection with HIV-1

Eight groups of either γ-irradiated nonobese diabetic/severe combined immunodeficient (NOD/SCID) BLT (NS-BLT) or NOD/SCID/IL2Rγc$^{-/-}$ BLT (NSG-BLT) mice were transplanted with individual human donor liver and thymus tissue expressing distinct human leukocyte antigen (HLA) haplotypes (Table 1), followed by injection of CD34$^+$ HSCs as previously described (*8*). Sufficient reconstitution with human immune cells (see Materials and Methods) usually occurred between 12 and 20 weeks after surgery, with NSG-BLT mice often reconstituting by 12 to 14 weeks after surgery, whereas NS-BLT mice required 16 to 20 weeks after surgery for sufficient reconstitution (table S1). This difference in reconstitution kinetics between NS-BLT and NSG-BLT mice has been previously described (*16*). Mice were then infected intraperitoneally with 1×10^5 $TCID_{50}$ (median tissue culture infectious dose) of HIV-1 JR-CSF molecular clone.

Evolution of HIV-1 in humanized BLT mice occurs predominantly within CD8$^+$ T cell epitopes

Because of the ability of HIV-1 to reproducibly escape from CD8$^+$ T cell responses in humans (*17*–*20*), we first looked for evidence of viral sequence evolution in BLT mice as a surrogate marker of immune activity. Full-length bulk sequencing of plasma-derived virus in three group M20 mice expressing HLA-A*01/A*02, -B*08/B*52, and -C*03/C*07 revealed considerable viral sequence diversity in each mouse by 6 weeks after infection (fig. S1). This early sequence evolution was predominated by nonsynonymous mutations, with 7 of these 25 nonsynonymous substitutions located within the 31 well-defined CD8$^+$ T cell epitopes restricted by the donor's HLA alleles (*21*). Because these 31 CD8 epitopes constitute only a small fraction of the total viral genome, early amino acid mutations in the BLT mice were thus found to arise disproportionately within the donor's restricted CD8 epitopes (odds ratio, 4.50; 95% confidence interval, 1.86 to 10.88; $P = 0.003$). In particular, reproducible viral evolution was observed in the epitopes Env A*01-RY9 (RRGWEILKY) and Nef C*03-AL9 (AAIDLSHFL), each of which evolved within two of the three M20 mice.

To conduct a more thorough analysis of early viral evolution, we cloned and sequenced a 908–base pair (bp) stretch spanning these two Env and Nef CD8 epitopes from six M20 mice at two time points between 6 and 12 weeks after infection. Although clones derived from the infecting JR-CSF viral stock were identical, clones derived from five of six mice exhibited a clear pattern of mutations across both the Env A*01-RY9 and the Nef C*03-AL9 epitopes (*21*). Viral evolution was also observed within a less commonly targeted Env A*02-LL9 (LLQYWSQEL) epitope (*22*, *23*) (Fig. 1A; see fig. S2A for both time points). Thus, viral escape from CD8$^+$ T cell responses appears to be rapid and highly reproducible, suggesting that multiple CD8$^+$ T cell responses are developing normally in humanized BLT mice after HIV-1 infection.

Viral escape pathways in genetically identical humanized BLT mice are reproducible

During viral escape from CD8$^+$ T cell responses in HIV-1 infection, the selection and outgrowth of specific mutations is influenced by the differential capacities of mutations to achieve efficient cytotoxic T lymphocyte (CTL) escape while minimizing the impact on viral protein structure and function (*24*–*27*). A closer examination of amino acid substitutions within the three evolving CD8 epitopes revealed that similar, albeit not identical, pathways of viral escape were used between the different M20 mice (Fig. 2A). In the Env A*01-RY9 epitope, amino acid positions 1 (P1) and 5 (P5) exhibited the highest frequencies of mutations, with a P1 arginine-to-histidine substitution predominating. In the Nef C*03-AL9 epitope, mutations were observed predominantly at P3, P4, and P5, with the P3 leucine and P4 asparagine notably representing 100% of the clones by week 12 in M20-25 and M20-16, respectively. Even in the subdominant Env A*02-LL9 epitope, evolution was limited to a P6 serine-to-glycine substitution in mice M20-16 and M20-27. Thus, similar forces appear to limit the possible pathways of viral escape from CD8$^+$ T cell responses in BLT mice.

HLA class I–associated evolution is consistent across mice expressing distinct HLA haplotypes

To further explore whether HLA-specific adaptations were reproducible across mice reconstituted from distinct human donor tissues but

Table 1. Genetic background and HLA haplotypes of humanized BLT mice. Each group of mice reconstituted with a single human donor's tissues and cells is designated by a group number, for example, M20; individual mice are further designated with a dash followed by the mouse number, for example, M20-07. Bold indicates groups from which sequence was examined. Underlined group numbers designate groups from which viral load data were determined. NS, NOD/SCID; NSG, NOD/SCID/IL2Rγc$^{-/-}$; ND, not determined.

Group	Genetic background	Number of mice	HLA-A*		HLA-B*		HLA-C*	
M16	**NS**	**1**	**01:01**	**36:01**	**35:02**	**49:01**	**04:01**	**07:01**
M20	**NS**	**6**	**01:01**	**02:01**	**08:01**	**52:01**	**03:03**	**07:01**
M24	**NS**	**8**	**02:01**	**26:01**	**15:01**	**27:05**	**01:02**	**03:04**
M30	NS	7	01:01	01:01	08:01	08:01	07:01	07:01
M31	**NSG**	**6**	**24:02**	**74:00**	**14:02**	**57:03**	**02:02**	**06:02**
M33	**NSG**	**1**	**02:01**	**24:03**	**ND**	**ND**	**03:03**	**07:02**
M47	NSG	7	02:06	24:02	15:02	40:01	03:04	04:03
M51	NSG	6	03:01	11:01	35:01	57:01	04:01	06:02

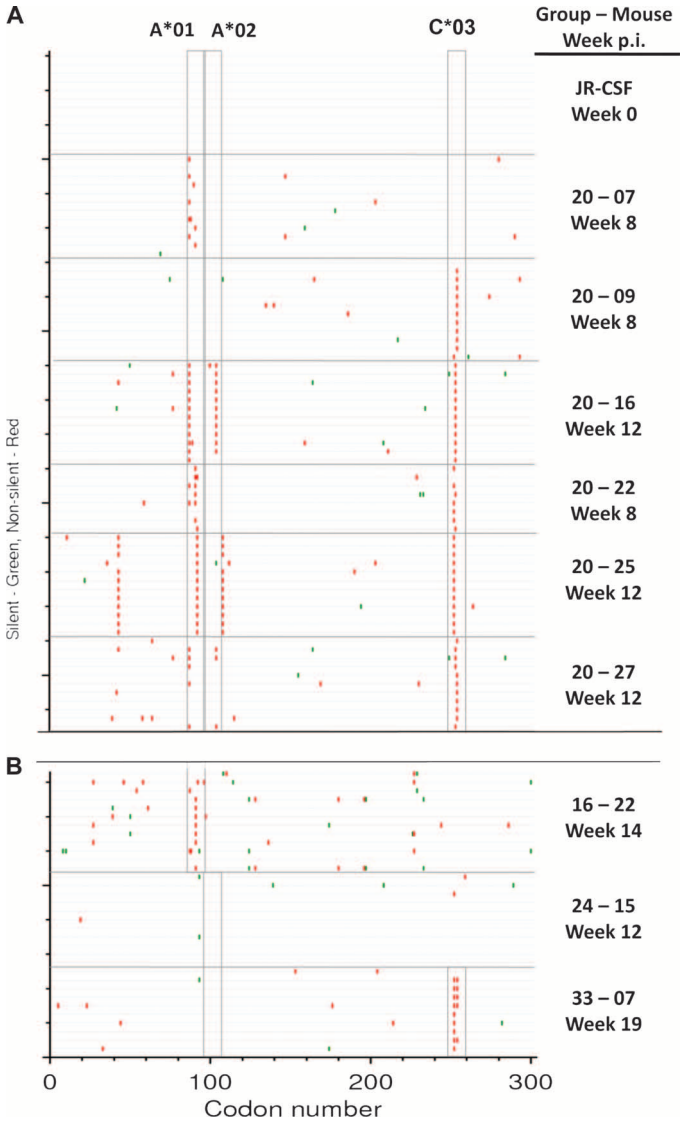

Fig. 1. Evolution of Env and Nef reveals mutations predominantly confined to CD8 epitopes. (**A** and **B**) HIV-1 was cloned and sequenced from (A) six M20 mice and (B) three mice from three additional reconstitution groups (M16, M24, and M33) sharing one or more HLA alleles with M20. Individual viral clones spanning a 908-bp fragment of Env and Nef were sequenced from each mouse indicated on the right. The Env and Nef open reading frames are shown as a single open reading frame. Each horizontal line represents a single clone. Each vertical colored dash represents evolution away from the infecting JR-CSF molecular clone, with nonsynonymous mutations in red and synonymous mutations in green (http://www.hiv.lanl.gov). Gray boxes define the location of the Env A*01-RY9, Env A*02-LL9, and Nef C*03-AL9 epitopes only in mice expressing the respective HLA allele. p.i., post-infection.

sharing the same HLA allele, we infected mice from three additional engraftments (M16-22, M24-15, and M33-07) sharing one or more of the M20 HLA alleles (Table 1). Viral evolution in the Env A*01-RY9 epitope was limited to the A*01-expressing mouse M16-22, whereas evolution in the Nef C*03-AL9 epitope was limited to one of the two C*03-expressing mice, M33-07 (Figs. 1B and 2B). Examination of the four-digit HLA typing revealed that although M33-07 expressed C*03:03 (identical to group M20), M24-15 expressed C*03:04, supporting the possibility that the restriction of this response may be specific to the expression of C*03:03.

In examining viral evolution across all infected mice, there was a statistically significant increase in the presence of nonsynonymous mutations in both the Env A*01-RY9 and the Nef C*03-AL9 epitopes in mice expressing the respective restricting HLA allele ($P < 0.001$), demonstrating the HLA-associated selection of these mutations. This effect remained significant for the Nef C*03-AL9 epitope even when mouse M24-15 (C*03:04) was included in the analysis ($P = 0.004$). These data demonstrate that the viral evolution observed is not random but rather restricted to the expression of a specific HLA class I allele.

Early HIV-1 sequence evolution is susceptible to stochastic events and is partially driven by human APOBEC3G/F activity

Early intrapatient HIV-1 sequence diversity, before the onset of adaptive immune pressures, is largely stochastic (*28*, *29*). This is mediated in part through the random error-prone nature of reverse transcription (RT) (*30*, *31*), as well as the cytidine deaminase hypermutation effects of the host factor APOBEC3G/F (*32*). Notably, 39% of all nucleotide mutations (112 of 286) in BLT mice within these first few weeks after infection reflected G-to-A substitutions, typical of APOBEC3G/F activity, with 21% of mutations (60 of 286) exhibiting characteristic signatures of human APOBEC3G/F hypermutation (*33*) (fig. S2B). This includes many of the mutations observed in the escaping CD8 epitopes, such as those in the Env A*01-RY9 epitope in M20-22 at week 6 and in the Nef C*03-AL9 epitope in M20-16 at week 12. These data support that the intrinsic ability of infected human $CD4^+$ T cells to inhibit viral replication capacity through human APOBEC3G/F proteins (*34*) shown in another humanized mouse model (*35*) is also conserved in this humanized BLT mouse model.

Epitope-specific $CD8^+$ T cell responses are detectable in BTL mice

The predominant selection of mutations in the Env A*01-RY9 and Nef C*03-AL9 epitopes suggested that these regions of the virus were under strong immune selection pressures. Using epitope-specific T cell lines stimulated for 2 to 3 weeks, we detected responses to each of these CD8 epitopes in M20 mice using interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assays (Fig. 3A) and confirmed the CD8 restriction of the Env T cell line through intracellular IFN-γ cytokine staining (Fig. 3B). Peptides representing the predominant mutations observed in each epitope were found to significantly impair immune recognition, with the most commonly observed P1 histidine and P3 leucine mutations in Env A*01-RY9 and Nef C*03-AL9, respectively, nearly completely impairing $CD8^+$ T cell recognition (Fig. 3, C and D). These data confirm that this rapid evolution of HIV-1 in the BLT mice reflects active evasion from human $CD8^+$ T cell responses.

BLT mice expressing HLA-B*57 restrict the same immunodominant CD8 epitopes seen in acutely infected humans

Particular HLA class I alleles, such as HLA-B*57, exhibit the ability to naturally control HIV-1 through the predominant targeting of the

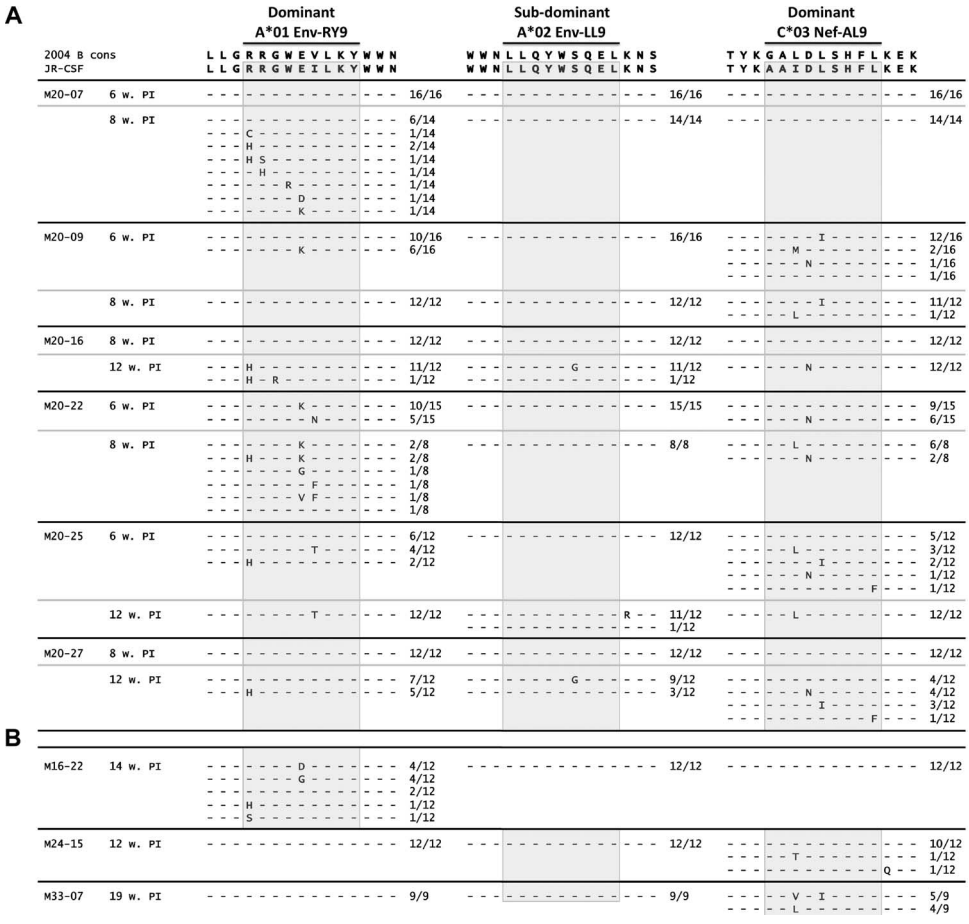

Fig. 2. Reproducible patterns of sequence evolution were observed in restricted CD8 epitopes. For each mouse at each time point, between 8 and 16 PCR clones spanning the three CD8 epitopes in Env and Nef were derived from a bulk sequencing reaction, cloned, and sequenced. The number of clones containing that sequence out of the total number of clones is indicated after the sequence. (**A** and **B**) Amino acid substitutions are shown for the three targeted CD8 epitopes in each mouse at two time points, in weeks post-infection (w. PI), for (A) the six M20 mice and (B) a single mouse from three additional reconstitution groups. The sequence of each epitope is indicated at the top for both the 2004 subtype B consensus sequence (http://www.hiv.lanl.gov) and the infecting JR-CSF strain. Gray boxes denote the location of the CD8 epitopes; dashes (-) denote amino acid residues matching JR-CSF.

12 after infection (Fig. 4B) and by MHC-I tetramer staining of the KF11-specific response at 12 weeks after infection (Fig. 4C).

Clonal sequencing of four M31 mice in these regions of Gag, Rev, and Nef at 10 weeks after infection revealed reproducible patterns of rapid viral evolution in the targeted Rev-KY10 and Nef-KF9 epitopes. In contrast, there was a notable lack of escape in the three Gag epitopes. We also observed reproducible evolution in all four mice within a short region of Vpu (Fig. 5). Although this region of Vpu does not contain any described CD8 epitopes for the HLA alleles expressed by the M31 mice, it is consistent with an HLA-B*57 binding motif [X-(ATS)-X-X-X-X-X-X-(FW)], and previous studies of human cellular immune responses have shown that two of four individuals who responded to this region of Vpu expressed HLA-B*57 (*44*, *45*). These observations are in line with the kinetics of viral escape observed in these epitopes in humans, with rapid escape in the Rev and Nef epitopes (*46*), but delayed escape from the more functionally constrained Gag epitopes (*45*, *47*–*49*). Additionally, there were few, if any, mutations observed within well-defined CD8 epitopes restricted by other HLA molecules expressed in the mice, suggesting that the immunodominance of HLA-B*57–restricted responses over other restricted responses (*43*, *50*) may be recapitulated in the BLT mice. Together, these data suggest that the Gag-specific $CD8^+$ T cell responses critical to the control of HIV-1 in B*57-positive humans and the sequence constraints that limit the ability of HIV-1 to rapidly escape from these responses are accurately reproduced in humanized BLT mice.

highly conserved Gag protein (*36*–*39*). To examine whether such critical immune responses restricted by protective HLA alleles in humans are also accurately reflected in humanized BLT mice, we used two groups of BLT mice (M31 and M51) reconstituted with tissues expressing HLA-B*57 (Table 1). Here, ex vivo IFN-γ ELISpot responses were detected with the addition of immortalized, isogenic B cell lines (BCLs) (fig. S3) (*40*–*42*). At 10 weeks after infection, M31 mice exhibited detectable ex vivo $CD8^+$ T cell responses against the immunodominant B*57-restricted epitopes Gag-IW9, -KF11, and -TW10 as well as Rev-KY10 and Nef-KF9 (*21*). Responses to each epitope were on the order of 200 to 600 spot-forming cells per million of peripheral blood mononuclear cells (PBMCs) (Fig. 4A), magnitudes that are in line with responses observed in HLA-B*57–positive, untreated, chronically infected humans (*43*). Development of these HLA-B*57–restricted responses was confirmed in the M51 mice by ELISpot assay at weeks 8, 10, and

Expression of HLA-B*57 in BLT mice is associated with stronger suppression of plasma viremia

Finally, we examined whether the ability of particular HLA alleles to exhibit control over HIV-1 might also be reproduced in this model. We first examined plasma viral loads in four groups of mice expressing common HLA alleles not associated with control of HIV-1 viremia (M20, M24, M30, and M47; 15 to 28 mice per time point). Viral loads in these mice were markedly consistent over time, exhibiting peak viral loads and set points greater than 1×10^5 copies/ml (Fig. 6) and a dip in viremia around 4 weeks after infection. We observed no significant difference in viral load set point among these mice. In contrast, viral loads in two independent groups of B*57-positive mice (M31 and M51; 9 to 12 mice per time point) exhibited significantly greater control of viral replication over the plateau stage of weeks 6 to 12, with mean viral loads greater than 0.6 $\log_{10}$ lower than in B*57-negative mice (42,000 versus 178,000

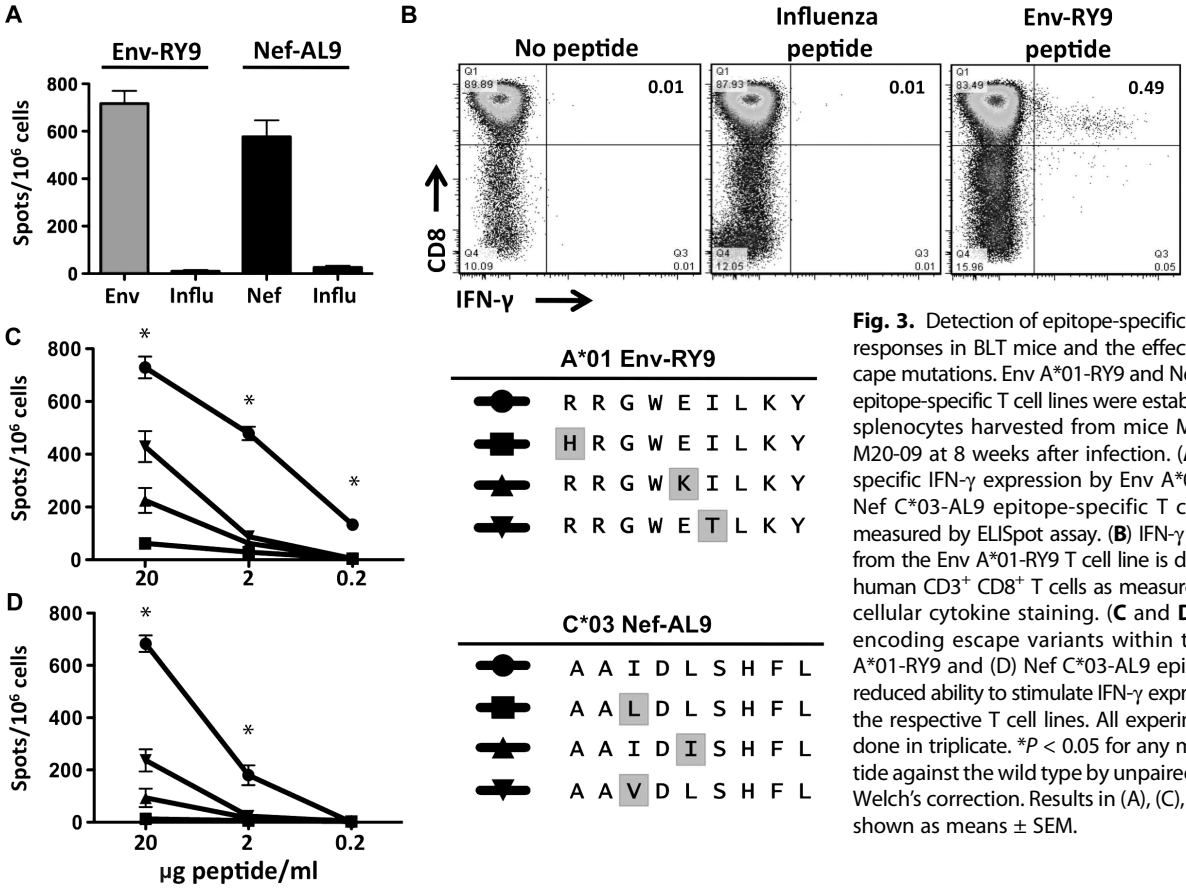

Fig. 3. Detection of epitope-specific CD8$^+$ T cell responses in BLT mice and the effect of CTL escape mutations. Env A*01-RY9 and Nef C*03-AL9 epitope-specific T cell lines were established from splenocytes harvested from mice M20-07 and M20-09 at 8 weeks after infection. (**A**) Antigen-specific IFN-γ expression by Env A*01-RY9 and Nef C*03-AL9 epitope-specific T cell lines as measured by ELISpot assay. (**B**) IFN-γ production from the Env A*01-RY9 T cell line is derived from human CD3$^+$ CD8$^+$ T cells as measured by intracellular cytokine staining. (**C** and **D**) Peptides encoding escape variants within the (C) Env A*01-RY9 and (D) Nef C*03-AL9 epitopes have reduced ability to stimulate IFN-γ expression from the respective T cell lines. All experiments were done in triplicate. *P < 0.05 for any mutant peptide against the wild type by unpaired t test with Welch's correction. Results in (A), (C), and (D) are shown as means ± SEM.

copies/ml; $P < 0.03$). These data support that the association of some protective HLA alleles, such as HLA-B*57, with significantly stronger control of HIV-1 viremia is recapitulated in humanized BLT mice.

DISCUSSION

The development of improved animal models capable of more accurately mimicking natural in vivo human immune responses to host pathogens would substantially accelerate biomedical research and facilitate the development of efficacious vaccines and therapeutics. Here, we explored the nature of human cellular immune responses to HIV-1 and viral evolution in the humanized BLT mouse model. Our data reveal that the specificity, functionality, and kinetics of HIV-1–specific CD8$^+$ T cell responses and viral escape pathways in BLT mice appear to closely resemble those seen in natural HIV-1 infection in humans.

Although cellular immune responses have been previously shown to develop in humanized mice (*7*–*10*, *51*, *52*), there has been a paucity of data regarding the specificity of theses responses at the epitope level. This, along with the limited data on the functionality and effectiveness of these responses against a human pathogen, has restricted our knowledge regarding the extent to which immune responses in these models accurately reflect those seen in humans. We were able to detect HIV-1–specific cellular immune responses in humanized BLT mice both through stimulation of epitope-specific T cell lines and directly ex vivo. The ability to detect responses at the epitope-specific level, combined with the viral escape data discussed below, indicates that this humanized BLT mouse model may be an attractive model to study the role of human epitope–specific responses to numerous pathogens as well as the factors governing the early induction and immunodominance patterns of CD8$^+$ T cell responses.

A particular strength of the humanized mouse model is its ability to generate multiple genetically identical animals. Because of the extensive genetic diversity within the human population, the study of immune responses and clinical outcomes in individuals expressing similar host genetics has proved critical to our understanding of the factors governing host immunity. Two studies of identical twins infected at the same time with the same source of HIV-1 have demonstrated that the kinetics and specificity of cellular immune responses to HIV-1 can be highly reproducible (*3*, *53*). Similar observations have been made in Mauritian macaques that exhibit low levels of MHC-I diversity (*54*). These studies reveal that there exist highly predictable patterns of CD8$^+$ T cell immunodominance during the acute phase of infection (*50*, *55*, *56*), which ultimately results in very similar patterns of viral escape from these responses (*3*, *57*). These data, although limited to only a few studies, have been critical to our understanding of the extent to which the specificity of early dominant CD8$^+$ T cell responses are dictated by both host and viral factors. Thus, the reproducibility of cellular immune responses and viral escape patterns seen among BLT mice with genetically identical immune systems suggests that this model has the potential to enable a more reliable interpretation of factors driving the specificity of early CD8$^+$ T cell responses and

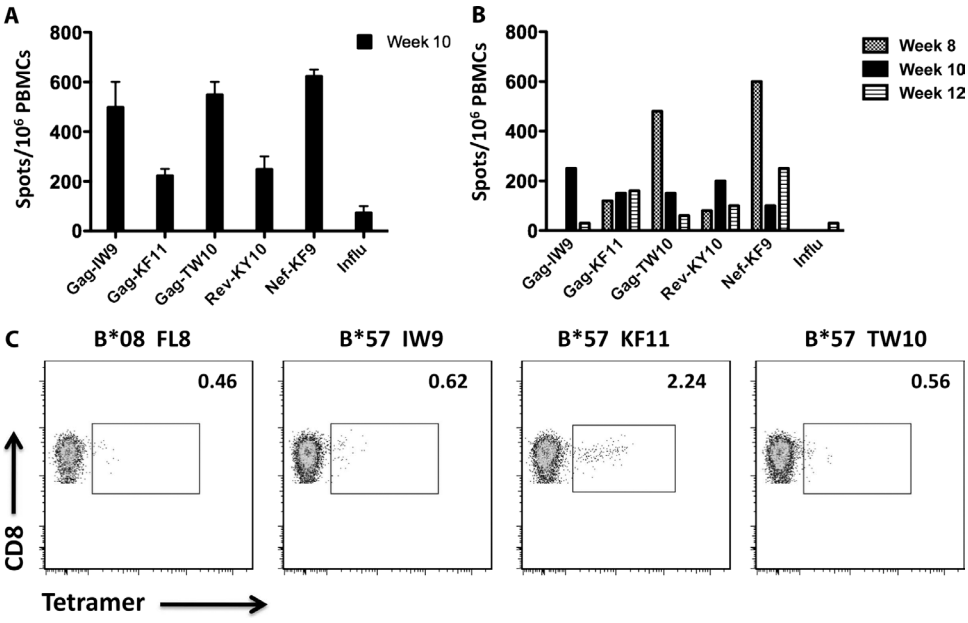

Fig. 4. Direct ex vivo detection of epitope-specific CD8$^+$ T cell responses in HLA-B*57–positive BLT mice. (**A**) Antigen-specific IFN-γ ELISpot responses were detected against five described B*57-restricted CD8 epitopes with PBMCs from M31 mice at week 10 after infection. (**B**) IFN-γ ELISpot responses to these epitopes were detected in PBMCs from M51 mice at weeks 8, 10, and 12 after infection. (**C**) MHC-I tetramer staining of human CD3$^+$, CD8$^+$ T cells from a mixture of spleen and liver/thymus organoid cells from an M51 mouse at week 12 after infection. The HIV-1 B*08-FL8 tetramer is included as a negative control. Results are representative of the two mice tested, each tested in triplicate. Results in (A) and (B) are shown as means ± SEM.

immunodominance patterns. Such knowledge may be critical to the design of vaccine antigens that would be capable of overcoming these natural immunodominance patterns to elicit responses that may be more critical to the control of HIV-1 (*58*, *59*).

Characterization of early genome-wide viral sequence evolution in BLT mice revealed that mutations were specific to well-defined CD8 epitopes and were reproducible across mice expressing the same restricting HLA allele. These data are consistent with studies in HIV-1 and SIV illustrating that the cellular immune response represents the major driving force of early viral sequence evolution (*20*, *46*, *60*). The Env A*01-RY9 and Nef C*03-AL9 epitopes that rapidly evolved in M20 mice represent the most immunodominant acute-phase responses for each of these alleles in humans (*55*). Rapid viral escape was also observed in the B*57-restricted Rev-KY10 and Nef-KF9 epitopes, both of which have been shown to quickly escape in humans before the commonly targeted B*57-KF11, -IW9, and -TW10 epitopes in Gag (*46*). These data suggest not only that humanized BLT mice can effectively mount multiple epitope-specific CD8$^+$ T cell responses very early after infection, but also that early CD8 epitope specificity, an attribute critical to the control of HIV-1 (*43*, *55*), may also be accurately reproduced in these mice. Furthermore, the escape pathways and kinetics observed in the escaping CD8 epitopes also resembled those seen in humans (*18*, *46*). Even the Env A*02-LL9 epitope, where slower viral escape was observed only subsequent to escape in the other two Env and Nef epitopes, appears to be rarely targeted in humans and likely represents a subdominant CD8 epitope (*22*, *23*). This pattern of evolution is similar to the normal shift in selection pressures toward secondary targets after viral escape from immunodominant epitopes during natural infection. Therefore, our data illustrate that the relative selection pressures on HIV-1 appear similar to those observed in acutely infected humans.

The mechanism associated with the ability of some HLA alleles to control HIV-1 infection appears to be due, at least in part, to the specificity of the CD8$^+$ T cell responses and their ability to dominate the early T cell repertoire. In general, Gag-specific CD8$^+$ T cell responses are broadly associated with the control of HIV-1 (*39*), and each of the protective HLA alleles B*57, B*27, and B*13 restricts dominant CD8$^+$ T cell responses against highly conserved regions of HIV-1 Gag (*24*, *61*, *62*). In humanized BLT mice expressing HLA-B*57, we were able to detect acute-phase responses to the three commonly targeted B*57-restricted epitopes TW10, IW9, and KF11 in Gag at magnitudes that are in line with those observed in a cohort of HLA-B*57–positive humans (*43*). In humans, the targeting of conserved regions of the virus such as Gag results in either delayed escape from these responses or the eventual selection of deleterious escape mutations that impair viral replication (*25*, *27*, *36*, *63*), translating into lower plasma viral loads and delayed disease progression (*64*–*66*). Similarly, in mice expressing HLA-B*57, we did not observe rapid development of escape mutations within the B*57-restricted Gag epitopes, and these mice exhibited a greater than 0.6 $\log_{10}$ lower level of viremia. This level of viral control in humans is associated with a significant reduction in the risk of progression to AIDS or death (*67*–*69*), and is also in line with the 0.7 $\log_{10}$ difference in viral set point associated with the expression of HLA-B*57 observed in a large cohort of chronically infected individuals (*70*).

Although our data support the use of the humanized BLT mouse model to complement studies in the SIV-infected rhesus macaque model, the small blood volumes available from these mice may limit more comprehensive immunological studies unless additional mice can be sacrificed. In addition, ex vivo PBMCs and even splenocytes from these animals responded suboptimally to stimulation with peptide antigen or even phorbol 12-myristate 13-acetate/ionomycin, which we reason may be due to poor antigen presentation or the lack of sufficient costimulatory signals. In light of this possible defect, it is important to highlight that their immune responses still provide sufficient pressure on the virus to drive the evolution of CTL escape mutations with similar kinetics to those seen in humans. Similarly, although we saw no correlation between the level of reconstitution of any measured cell type with viral loads and the rate of viral escape between mice, it is possible that subtle differences in reconstitution levels at the time of challenge could affect individual response rates. In addition, in our study, mice were infected intraperitoneally with a high dose of HIV-1 to guarantee infection of all mice. The minimal dose of HIV-1 required

A

		B*57 Gag-IW9	B*57 Gag-KF11	B*57 Gag-TW10
2004 B cons		H Q A I S P R T L N A W V K V	I E E K A F S P E V I P M F A A L	A G T T S T L Q E Q I G W M T N
JR-CSF		H Q A I S P R T L N A W V K V	I E E K A F S P E V I P M F A A L	A G T T S T L Q E Q I G W M T N
M31-03	10 w. PI	15/15	15/15	15/15
M31-07	10 w. PI	15/16	16/16	15/16
		M 1/16		A 1/16
M31-09	10 w. PI	16/16	16/16	16/16
M31-12	10 w. PI	15/15	15/15	15/15

B

		B*57 Rev-KY10	B*57 Nef-KF9	Predicted B*57 Vpu-VW10
2004 B cons		E L L K T V R L I K F L Y Q S N	M T Y K G A L D L S H F L K E	A L V V A G I I A I I V W S I V
JR-CSF		D L L K T V R L I K F L Y Q S S	M T Y K A A I D L S H F L K E	A L V V A G I I A I I V W S I V
M31-03	10 w. PI	7/17	9/12	5/17
		A 5/17	L 3/13	V 5/17
		L 4/17		3/17
		A 1/17		T 3/17
				L V 1/17
M31-07	10 w. PI	9/12	12/12	6/12
		S 3/12		I 5/12
				T 1/12
M31-09	10 w. PI	8/11	12/12	V 4/10
		F 2/11		T 4/10
		P 1/11		L 1/10
				1/10
M31-12	10 w. PI	A 4/10	V 6/7	5/10
		L 3/10	N 1/7	V 5/10
		G L 1/10		
		A 1/10		
		V 1/10		

Fig. 5. Differential immune escape kinetics and reproducible mutation patterns within HLA-B*57–restricted CD8 epitopes in BLT mice. (**A**) Clonal sequences were derived from plasma viral RNA from four M31 mice at 10 weeks after infection. (**B**) Escape mutations in the Rev, Nef, and Vpu epitopes were observed in each of the M31 mice, although no evolution was observed in any of the three Gag epitopes. The Vpu region has not been previously described as a targeted epitope but is predicted to bind HLA-B*57 [SYFPEITHI; (*73*)].

to establish efficient levels of infection by mucosal routes or through multiple low-dose exposures, which have recently proven critical for gauging vaccine efficacy in the SIV-infected macaque model, remains to be determined. Finally, although this study was focused on HIV-1–specific $CD8^+$ T cell responses, it will be of great interest for future studies to determine the extent to which other arms of the immune system, including $CD4^+$ T cell responses, neutralizing antibodies, and natural killer cell responses, recapitulate those of natural HIV-1 infection.

The ability of this platform to also generate groups of genetically identical animals could greatly facilitate identification of the host and viral factors associated with the induction of human immune responses as well as mechanisms of immune control of HIV-1. The ability to vary a single experimental component (such as viral strain, vaccine antigen, or adjuvant), adoptively transfer T cells, and deplete immune subsets, all while examining the same HIV-1–specific responses observed in subjects, would enable a more precise identification of immune correlates of control of HIV-1 in humans. Furthermore, the availability of genetically identical animals should provide additional power for vaccine studies because of removal of the heterogeneous host genetic component that can often confound vaccine studies. In particular, this model may lend itself well to the study of $CD8^+$ T cell immunodominance whereby manipulation of immunogens to focus responses toward CD8 epitopes that are relatively constrained in their ability to evolve escape mutations is believed to be critical to the control of HIV-1. Such studies could facilitate the design of vaccine approaches that induce more robust, longer-lived immune responses that provide better protection (*58*).

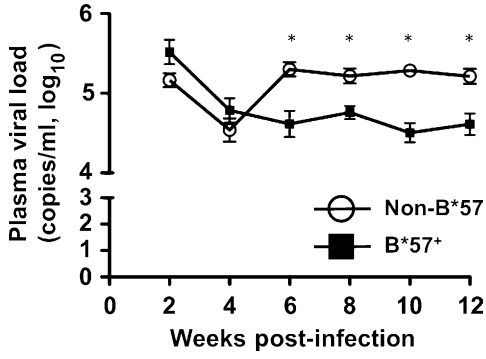

Fig. 6. HLA-B*57–positive mice exhibit enhanced control of viral replication. Open circles denote viral loads (mean RNA copies/ml plasma ± SEM) from four groups of mice that do not express protective HLA alleles (the number of mice at each time point, in order, is 32, 28, 19, 16, 14, and 14). Closed squares denote viral loads from two groups of mice expressing HLA-B*57 (the number of mice at each time point, in order, is 12, 12, 12, 9, 9, and 8), which exhibited significantly lower viral loads at and beyond 6 weeks of infection (*$P < 0.03$ for B*57 for both groups when combined or compared separately by the Mann-Whitney-Wilcoxon test).

In summary, we have shown that humanized BLT mice are capable of rapidly mounting HIV-1–specific $CD8^+$ T cell responses that reflect the epitope specificity and kinetics observed during HIV-1 infection of humans. Moreover, the relative ease by which HIV-1 escapes some but not all $CD8^+$ T cell responses in BLT mice also appears to be conserved. Finally, the expression of the protective HLA-B*57 allele in BLT mice enabled the mounting of responses against conserved regions of HIV-1 Gag that are associated with greater control of viral replication in humans, and our data now indicate that this association is conserved in these mice. Together, these results suggest that the humanized BLT mouse model represents a fundamental advancement in the ability of small-animal models to accurately reproduce human-specific immune responses to human pathogens, and supports the ability of this model to complement pathogenesis and vaccine studies for HIV-1, as well as a number of other human pathogens.

MATERIALS AND METHODS

Animals and infections

NOD/SCID and NOD/SCID/IL2R$\gamma^{-/-}$ mice (The Jackson Laboratory) were housed in a pathogen-free facility at Massachusetts General Hospital, maintained in microisolator cages, fed autoclaved food and water, and reconstituted with human tissue as described (*8*). Mice were generally considered reconstituted if greater than 40% of cells in the lymphocyte gate were human $CD45^+$ and greater than 30% of these human $CD45^+$ lymphocytes were human $CD3^+$. We performed genotyping of the *HLA* locus using PCR-SSOP (polymerase chain reaction–sequence-specific oligonucleotide probing) and PCR-SBT (sequence-based typing). Viral stocks of the JR-CSF HIV-1 molecular clone were produced through transfection of human embryonic kidney (HEK) 293T cells and titered as described (*63*). Mice were infected intraperitoneally with 1×10^5 $TCID_{50}$ of JR-CSF HIV-1. Every 2 weeks after infection, about 200 μl of blood was obtained through puncture of the retro-orbital sinus or submandibular vein for isolation of plasma virus.

RNA isolations and viral load quantitation

Viral RNA was isolated with the QIAamp Viral RNA Mini Kit (Qiagen). Plasma viral loads were determined by quantitative RT-PCR with the QuantiFast SYBR Green RT-PCR kit (Qiagen) as described (*63*).

Viral sequencing

Plasma viral RNA was subjected to RT-PCR with the SuperScript III One-Step RT-PCR System (Invitrogen). PCR products were amplified with nested PCR with two overlapping sets of primers covering the entire HIV-1 proteome. Products were ligated into pCR2.1 (Invitrogen), and individual clones were sequenced with the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Highlighter plots were produced with tools from the HIV Molecular Database at the Los Alamos National Laboratory (http://www.hiv.lanl.gov). Viral sequences are available under accession numbers JX073143 to JX073254.

Generation of HIV-specific T cell lines and BCLs

Epitope-specific T cell lines were produced by pulsing 2 million splenocytes with specific peptides (12 μg/ml) in the presence of 10 million irradiated allogenic PBMCs and grown in RPMI with 10% fetal calf serum (FCS) and interleukin-2 (IL-2) (50 U/ml) (*71*). BCLs were produced from PBMCs via infection with Epstein-Barr virus from the supernatant of the B95-8 cell line (American Type Culture Collection: CRL-1612) in the presence of cyclosporine A (*72*).

Measurement of $CD8^+$ T cell responses by IFN-γ ELISpot assay

T cell lines were tested for activity with an IFN-γ ELISpot assay (*44*). For direct ex vivo IFN-γ ELISpot assays, isogenic BCLs at 2 million cells/ml were pulsed with peptide at a final concentration of 100 μg/ml and incubated at 37°C and 5% CO_2 in a humidified incubator for 1 hour before assay. Then, 200,000 peptide-pulsed BCLs were added to 100,000 cells in a 200-μl volume on a 96-well Immobilon-P filter plate (Millipore) and incubated at 37°C for 48 hours before colorimetric development. Negative controls for ELISpot assays were incubated with a nonspecific influenza peptide, influenza peptide–pulsed isogenic BCLs, or HLA-mismatched allogenic BCLs pulsed with HIV-1 peptide.

Intracellular cytokine staining and MHC-I tetramer staining

IFN-γ production from T cell lines was measured by flow cytometry. T cells (1×10^6) were stimulated with the corresponding specific peptides Env A*01-RY9 or Nef C*03-AL9 (20 μg/ml). Negative controls were stimulated with an influenza peptide. Splenocytes were incubated with peptide for 1 hour, brefeldin A was added, and cells were incubated for an additional 4 hours. Cells were stained with antibodies against human CD3, CD4, and CD8 for 20 min, then fixed with Perm A and permeabilized with Perm B (BD Biosciences), and stained with antibodies against IFN-γ for 20 min. Samples were analyzed by flow cytometry. For MHC-I tetramer staining of both the T cell line and the mix of cells from the spleen and thymic organoid from a mouse, cells were stained with antibodies against CD8 and antigen-specific MHC-I tetramers for 30 min. Here, spleen and thymic organoid were pooled to increase the number of cells obtained from the mouse. Cells were washed and fixed in 1% paraformaldehyde and analyzed by flow cytometry.

Statistical analysis

The Mann-Whitney-Wilcoxon test was used to determine significant differences between viral loads. Fisher's exact test was used to determine whether the number of nonsynonymous mutations arising within well-defined $CD8^+$ CTL epitopes restricted in the M20 mice (*21*) was significantly higher than the total number of mutations found outside these confirmed epitopes. An unpaired Student's *t* test was used to compare ELISpot results between wild-type and mutant peptides.

SUPPLEMENTARY MATERIALS

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Fig. S1. Viral evolution in BLT mice at 6 weeks post-infection (p.i.) arises within described CD8 epitopes.

Fig. S2. Highlighter plot illustrating viral evolution dynamics and the predominance of APOBEC3G/F activity.

Fig. S3. The presence of isogenic BCLs rescues antigen-specific IFN-γ expression by $CD8^+$ T cells from BLT mice.

Table S1. Individual reconstitution levels of human and mouse lymphoid subsets.

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