Severe human Lassa fever is characterized by non-specific T-cell activation and lymphocyte homing to inflamed tissues.

Keywords: Lassa virus, T cells, T-cell homing, pathogenesis

Corresponding author: César Muñoz-Fontela, Bernhard Nocht Institute for Tropical Medicine, Bernhard Nocht Strasse 74, 20359 Hamburg, Germany. Email: munoz-fontela@bnitm.de.
Abstract
Lassa fever (LF) is a zoonotic viral hemorrhagic fever caused by Lassa virus (LASV), which is endemic to West African countries. Previous studies have suggested an important role for T cell-mediated immunopathology in LF pathogenesis, but the mechanisms by which T cells influence disease severity and outcome are not well understood. Here we present a multiparametric analysis of clinical immunology data collected during the 2017-2018 Lassa fever outbreak in Nigeria. During the acute phase of LF we observed robust activation of the polyclonal T-cell repertoire, which included LASV-specific as well as antigenically-unrelated T cells. However, severe and fatal LF were characterized by poor LASV-specific effector T-cell responses. Severe LF was also characterized by the presence of circulating T cells with homing capacity to inflamed tissues, including the gut mucosa. These findings in LF patients were recapitulated in a mouse model of LASV infection, in which mucosal exposure resulted in remarkably high lethality compared to skin exposure. Taken together, our findings indicate that poor LASV-specific T-cell responses and activation of non-specific T cells with homing capacity to inflamed tissues are associated with severe LF.

Importance
Lassa fever may cause severe disease in humans, in particular in endemic areas such as Sierra Leone and Nigeria. Despite its public health importance, the pathophysiology of Lassa fever in humans is poorly understood. Here we present clinical immunology data obtained in the field during the 2018 Lassa fever outbreak in Nigeria indicating that severe Lassa fever is associated with activation of T cells antigenically unrelated to Lassa virus as well as with poor Lassa virus-specific effector T cell responses. Mechanistically, we show that these bystander T cells express defined tissue homing signatures that suggest their recruitment to inflamed tissues and a putative role of these T cells in immunopathology. These findings open
a window of opportunity to consider T cell targeting as a potential post-exposure therapeutic strategy against severe Lassa fever, a hypothesis that could be tested in relevant animal models such as non-human primates.

Introduction

Lassa fever (LF) is a severe viral disease endemic to West African countries such as Nigeria, Guinea, Liberia and Sierra Leone. The causative agent is Lassa virus (LASV; species *Lassa mammarenavirus*; family *Arenaviridae*), a single-stranded bi-segmented RNA virus. LF is a zoonotic disease and seasonal outbreaks are caused by multiple spillover events from the virus’s rodent host, the multimammate rat *Mastomys natalensis*, into humans (1). In hyperendemic regions such as southern Nigeria and Sierra Leone, LASV may cause up to 300,000 estimated LF cases and 5,000 deaths annually (1, 2). Although there are limited epidemiological data on LASV transmission, human infection is thought to occur mainly through contact with rodent excreta, which may involve consumption of contaminated food, inhalation of particles containing infectious virus and skin contact with infected rodent body fluids (2, 3). Contact with infectious body fluids is also the main route of human-to-human transmission either in the household or during nosocomial outbreaks (4).

A LF outbreak is defined as a significant increase of cases over the baseline number of cases that occur during the regular Lassa season. For reasons that are not well understood, outbreaks take place in endemic areas at random intervals. In 2018, the Nigerian LF season was characterized by the largest ever recorded number of cases in the country (5). Our laboratory, located at the Nigerian reference center for Lassa fever at the Irrua Specialist Teaching Hospital (ISTH) was engaged in outbreak control, diagnostics and patient treatment. In this context, we set up an operational research protocol to better understand host factors driving disease severity.
In a previous study, we demonstrated that depleting T cells during the course of LF in a mouse model was sufficient to rescue mice from death (6), and severe LF disease was achieved in a mouse model expressing a human HLA-A2 transgene (7). However, these findings were restricted to infection models and their relevance in humans was not known. There is substantial evidence that, in humans, as well as non-human primates, early T-cell activation is correlated with recovery and viral clearance (8, 9). Furthermore, fatal LF has been associated with lymphopenia in previous studies (10). However, studies on human immunology during acute disease are rare and the overall role of T cells in Lassa fever is poorly understood. During viral infections, there are several mechanisms by which T cells can influence pathogenesis including activation of bystander T cells, activation of memory T cells due to latent virus reactivation, production of pro-inflammatory cytokines and homing into inflamed tissues where T cells can directly cause tissue damage by killing infected target cells (11, 12).

Bystander activation of T cells independent of T-cell receptor (TCR) engagement, as well as their reactivation by latent virus infection, has been associated with exacerbation of pathogenesis in certain infections including Lyme disease, leishmaniasis, HIV, dengue fever and Ebola virus disease (13-15). Antigenically unrelated T-cell activation during acute infection occurs as a consequence of reactivation of latent virus infections, as well as through the effect of an inflammatory milieu, where antigen-independent T cells become effector T cells due to cytokine stimulation or through engagement of pattern-recognition receptors (PRRs) (11, 13). Massive recruitment of virus-specific as well as non-specific T cells to skin and gut has been previously correlated with the immunopathology caused by infectious diseases such as dengue, rotavirus and herpesvirus infection (16-18). An additional consequence of non-specific T-cell activation is the initiation of anti-inflammatory mechanisms, upregulation of T-cell immune checkpoints and/or induction of T-cell...
anergy, which may cause an overall reduction of pathogen-specific T-cell responses (12, 19). However, the putative role of non-specific T-cell activation in LF pathogenesis has not been yet investigated.

Both antigen-specific as well as non-specific T cells can be recruited into inflamed tissues via expression of defined ‘homing’ markers such as cutaneous lymphocyte antigen (CLA) or lymphocyte Peyer’s patch adhesion molecule (LPAM-1, or alpha(4)beta(7) integrin), which bind E-selectin and mucosal addressin cell adhesion molecule (MadCAM)-expressing cells, respectively, (20-22) and thereby drive the recruitment of T cells to the skin and gut mucosa. Thus, through their expression of tissue-specific homing molecules, activated T cells are able to leave the blood and lymphatic systems and migrate into specific organ tissues. Consequently, infectious diseases with one primary route of transmission or strong tissue tropism are characterized by the presence of peripheral blood T cells with defined T-cell homing signatures (17, 21, 23). Thus, understanding T-cell homing patterns in LF could provide insight into migratory patterns of T cells during the acute phase of disease and its possible implications in immunity and immunopathology.

Here we analyzed T-cell responses from patients diagnosed with LF during the 2017-2018 Lassa fever outbreak in Nigeria, and show that antigenically-unrelated T-cell activation and poor LASV-specific effector T-cell responses are associated with severe disease. Moreover, severe LF was characterized by the presence of T cells with homing signatures to mucosal tissues. These findings suggest that recruitment of virus-specific and non-specific T cells with poor capacity for virus clearance to inflamed tissues may be an important component of LF pathophysiology.
Results

Cohort description and clinical manifestations of LF.

The LF cases included in this study were admitted to the Lassa ward at ISTH in Edo state, Nigeria between January 2017 and June 2018. In total, n=214 patients were diagnosed as positive for LF via RT-PCR and enrolled into our study, out of which 17% succumbed to the disease (Fig. 1A). Most of the enrolled patients came from the neighboring states of Edo and Ondo in southwestern Nigeria. Demographically, 62% of patients were male and 33% of the patients were between 21 and 30 years of age (Fig. 1B, C). Upon admission, all patients had fever and more than 50% reported weakness, headache and gastrointestinal manifestations such as abdominal pain and vomiting (Fig. 1D). Samples were collected from patients longitudinally during their stay in the Lassa ward, coincident with the acute phase of LF. An initial functional analysis of the T-cell response during acute LF was performed at ISTH (n=13 patients) as well as a first assessment of T-cell phenotype markers (n=36 patients). Further multiparametric assessment of T-cell phenotype was performed on samples from n=22 patients that were cryopreserved and shipped to the biosafety level 4 (BSL-4) laboratory at the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany (Fig. 1E). Patients were classified as fatal, or survivors based on outcome. Among survivors, patients were classified retrospectively as severe cases if their levels of serum aspartate aminotransferase (AST) were higher than 300 U/L at any point during disease, while cases were defined as mild if AST levels were lower than 300 U/L throughout the course of LF. Upon admission, patients provided a self-reported days post-symptom onset (DPO) estimate, which were comparable across mild, severe and fatal cases (Fig. 1F). For flow cytometry analysis, sample viability was assessed in relation to corresponding AST serum levels, a biomarker of disease severity (Fig. 1G).
Acute Lassa fever induces non-specific T-cell activation.

To characterize the overall T-cell response during human LF, we examined the presence of virus-specific T cells and antigenically-unrelated T cells during the acute phase of infection, as well as shortly after discharge from the Lassa ward. Using MHC class I tetramers bound to the HLA-A2 restricted LLGFTWTL LASV peptide (8), we observed that the generation of epitope-specific CD8 T cells was limited during the acute phase of the disease but increased significantly after patient discharge (Fig. 2A). However, evaluation of the activated T-cell compartment defined as CD38+ or CD38+ HLA-DR+ cells (24, 25) indicated the opposite trend, with a significantly higher percentage of activated T cells observed during the acute phase compared to the post-recovery phase (Fig. 2B). LASV-specific CD8 T cells also expressed activation markers during acute infection, but the overall frequency of activated cells (up to 22% of total CD8 T cells) (Fig. 2B), indicated polyclonal CD8 T-cell activation that could include LASV-specific and non-specific T cells. Previous studies in HIV patients identified antigenically-unrelated CD8 T cells expressing high levels of CD38 (26). Following this criterion, we utilized Epstein-Barr virus (EBV)-specific MHC class I tetramers to determine whether antigenically-unrelated T cells were also activated during acute LF. We observed a discrete subset of EBV-specific CD8 T cells that expressed CD38 during LASV infection but not in healthy controls, suggesting that non-LASV specific T-cell activation indeed occurred during acute Lassa fever (Fig. 2C).

To further determine whether non-specific T-cell activation occurred during LASV infection in vivo, we utilized a mouse model of severe infection previously developed in our laboratory that allows for functional studies of T cells during LF (6). We engineered a mixed bone marrow chimera using recipient type I interferon (IFN) receptor knockout mice (IFNAR−/−), transplanted at a 1:1 ratio with bone marrow progenitor cells from wild type (CD45.1) mice and TCR-transgenic mice designed to
recognize the MHC class I-restricted ovalbumin (OVA) peptide SIINFEKL (hereafter referred to as OT-I mice) (Fig. 2D). To track the activation of bystander T cells in this model we searched for OT-I-specific CD8$^+$ CD44$^+$ T cells as previously described (11). Infection of these mixed chimeras with LASV via intranasal (i.n.) inoculation resulted in strong activation of the polyclonal wild type T-cell repertoire in which roughly 90% of cells in blood expressed CD44 nine days after infection. The majority of this activated subset also downregulated the expression of the lymphoid homing marker CD62L, suggesting recruitment of activated T cells into inflamed peripheral tissues (Fig. 2E, F). Although to a lesser extent, around 40% of OT-I T cells also upregulated CD44 indicating non-specific T-cell activation (Fig. 2F). In this case, 25% of the total OT-I CD8 T-cell pool also downregulated CD62L, indicating the presence of a subset of bystander T cells with the capacity for recruitment to LASV-infected tissues.

Taken together, our results indicated that antigenically-unrelated T-cell activation takes place during LF in humans as well as in a mouse model of infection. In the latter, our data suggest that a subset of bystander cells have capacity to infiltrate LASV-infected tissues during infection.

Severe LF is characterized by poor LASV-specific T-cell responses.

To better understand the dynamics of T-cell activation during acute LF, we sought to investigate the clonality and kinetics of the T-cell response in patients during their stay at the Lassa ward. To this end, we performed TCR sequencing in peripheral blood samples of surviving and non-surviving patients from the onset of symptoms to death or recovery. Evaluation of T-cell repertoire diversity showed no significant differences between fatalities and survivors in the acute phase of the disease, suggesting that similar levels of clonal T-cell expansion occur in fatal and non-fatal
However, we detected differences in the kinetic trends of the clonal T-cell expansion between survivors and fatals. In survivors, the frequency of hyperexpanded T-cell clones, that is, clones that dominate the overall T-cell response, increased towards the end of the patient’s stay at the ward in coincidence with virus clearance and discharge. Conversely, non-survivors showed the opposite trend, with hyperexpanded clones dominating the response at early time points after onset (Fig. 3B). These results suggested that, in fatal LF, the early antiviral response was dominated by T-cell clones with poor ability to clear virus.

One of the consequences of non-specific T-cell activation is that it leads to a regulatory environment that can prevent the immune system from mounting an adequate response (19). Thus, we hypothesized that, in severe LF, the presence of antigenically-unrelated cells could lead to diminished LASV-specific responses. To test this hypothesis, we evaluated the LASV-specific effector T-cell profiles in samples from patients showing different degrees of disease severity as measured by viremia and AST levels in blood. We utilized a LASV nucleoprotein (NP)-derived peptide pool to restimulate peripheral blood T cells, and assessed their capacity to degranulate (by expression of surface CD107a) and produce effector cytokines such as TNF-$\alpha$ and IFN-$\gamma$. Within a sample size of n=26 samples collected from n=13 patients, 11.5% of samples showed a multifunctional T-cell response characterized by degranulation and production of both cytokines. However, we observed that, in 15.4% of samples, T cells did not respond at all to re-stimulation with LASV antigen (Fig. 3C). In order to stratify these data across different ranges of disease severity, we evaluated whether there was a relationship between T-cell effector functions and biomarkers of severity such as the levels of serum AST and viremia. We observed that, in samples with corresponding serum AST levels greater than 200 U/L, T cells did not show any effector functions, namely, they neither degranulated nor produced...
any cytokines. IFN-γ production was greater in T cells from samples with corresponding AST levels between 50-200 U/L, while TNF-α production was observable in that same range or lower (Fig. 3D). Similarly, T-cell effector functions were observable only in samples with linked low levels of viremia, namely high cycle threshold (Ct) values, indicating an association between high virus loads, liver pathology and poor T-cell function (Fig. 3E).

These findings suggested that severe LF was associated with the expansion of T-cell clones with poor effector functions, resulting in lower capacity to control virus replication.

**Fatal LF is characterized by T-cell homing to inflamed tissues**

The identification of non-specific T cells and poorly functional effector T cells in severe LF prompted us to evaluate whether these cells had the capacity to migrate to inflamed tissues. Thus, we investigated T-cell homing patterns in activated T cells during acute LF and their relationship with disease severity. In a first screening in Nigeria, we evaluated the presence of homing markers in the activated T-cell compartment of n=36 patients. We evaluated the presence of CLA as marker of skin homing (n=61 samples), integrin-β1 (ITGB1) and ITGA4 (n=25 samples) as general markers of lymphocyte homing to inflamed tissues, ITGB7 (n=25 samples) which, together with ITGA4, forms LPAM-1 driving T-cell homing to the gut, and CCR3 (n=16 samples) as a homing marker for the respiratory mucosa (16, 21, 27). The data obtained in this screening indicated that, during acute LF, the activated T-cell compartment was dominated by cells with homing capacity to inflamed tissues, the gut mucosa and, to a lesser extent, to the skin and respiratory tract (Fig. 4A). To gain further insight into the relationship between T-cell homing and LF severity, we performed additional immunophenotyping of activated CD8 and CD4 T cells in acute cryopreserved LF samples using multiparametric flow cytometry panels (n=22...
patients, 54 samples). We evaluated the levels of expression of markers of T-cell homing to skin, mucosae and lymphoid tissue in activated T cells, and determined sample clustering via principal component analysis (PCA). We observed the presence of four defined clusters, characterized by differences in disease outcome. Samples from fatal Lassa fever cases were predominantly found in clusters 1 and 2 (67% and 60% CFR respectively) while survivors formed the majority of cases grouped in clusters 3 and 4 (4% and 0% CFR respectively) (Fig. 4B, C). These results suggested an association between T-cell homing signatures and LF outcome.

Overall, our data indicated that the acute phase of LF was characterized by activated T cells with homing capacity to inflamed tissues. These lymphocyte homing signatures drive patient clustering in groups with marked differences in disease severity.

**Unique T-cell populations in LF patients with different degrees of severity**

To better understand the relationship between T-cell homing and LF severity we next sought to dissect the homing signatures of the polyclonal T-cell response in severe, mild and fatal LF. Due to the fact that T-cell-mediated immunity is polyclonal, the overall response reflects the combined action of different T-cell subsets with overlapping and non-overlapping functions. We reasoned that, within the polyclonal repertoire that characterized the activated T-cell compartment during acute LF, there might be virus-specific as well as non-specific T cells with different homing signatures. Thus, we utilized multidimensional flow cytometry followed by t-distributed stochastic neighbor embedding (t-SNE) analysis to investigate the presence of unique T-cell subsets and their homing signatures in fatal, mild and severe LF cases (Fig. 5A). Samples from fatal LF cases were enriched in activated CD8 and CD4 T-cell subsets with a central memory phenotype (CCR7+ CD45RA−) suggesting perhaps activation of T-cell clones not specific for LASV. Clones
expressing high levels of ITGA4 were also dominant in both the CD4 and CD8 compartment. In the latter, we also observed clones that expressed CCR3, which suggested migration of CD8 T cells to the respiratory tract (Fig. 5B). Among survivors, severe cases showed a CD8 T-cell clonal space that was dominated by CD45RA+ effector T cells that either retained CCR7 expression or not. Also, severe cases showed the highest presence of CD8 T-cell clones with homing capability to the gut (ITGA4+ ITGB7+). Interestingly, samples from this cohort also harbored clones with high expression levels of CLA in both the CD8 and CD4 T-cell compartments. These results indicated that severe LF is characterized by effector T cells capable of migrating to the gut and skin. Finally, samples from mild cases that maintained low levels of viremia and serum AST throughout the acute phase of disease showed low expression levels of CCR7 in most of the T-cell subsets and overall low levels of CD45RA, consistent with an effector memory phenotype. In mild cases we observed clones with strong expression of ITGA4, suggesting migration to sites of but overall low levels of tissue-specific homing factors in CD8 T cells. We further observed gut and skin signature in CD4 T cells.

Taken together, our findings suggested that fatal LF is associated with reactivation of central memory T-cell clones with the capacity to migrate to sites of inflammation such as the respiratory mucosa. Both severe and mild cases showed the presence of effector T cells with the capacity for homing to the gut and skin, and revealed differences in homing signatures between CD8 and CD4 T cells.

Mucosal exposure to LASV results in high lethality in a mouse model

Our findings in patients strongly suggested that fatal and severe LF was associated with the migration of activated T cells, particularly CD8 T cells, to infection sites such as the gut and respiratory mucosae. Because T-cell homing is imprinted by antigen-presenting cells located primarily at the site where T cells first encounter antigen (23,
we reasoned that the observed differences in disease severity could be related to some extent to the route of infection. To test this hypothesis we utilized IFNAR\textsuperscript{B6} bone marrow chimeras (IFNAR\textsuperscript{−/−} reconstituted with wild type (CD45.1) bone marrow) in which we previously showed that intraperitoneal infection of LASV resulted in 100\% lethality (6). To test the effect of the inoculation route in this model we inoculated mice with 1000 focus-forming units (FFU) of LASV via intranasal (i.n.), intradermal (i.d) or oral (p.o) inoculation. Intranasal infection of IFNAR\textsuperscript{B6} chimeras with LASV resulted in 100\% lethality and the shortest time to death after infection (8-10 days). Oral administration of LASV also resulted in high lethality (60\%) in this model, even though the time to death was delayed to nearly 20 days post-infection in some subjects. Conversely, intradermal inoculation of LASV resulted in 25\% lethality (Fig. 6A). Furthermore, mice that were inoculated either intranasally or orally with LASV lost weight over the course of the disease and had fever, high levels of circulating AST, and high levels of viremia. Intradermal inoculation resulted in a milder disease phenotype characterized by low serum AST, the absence of fever and low levels of viremia (Fig. 6B-E).

In agreement with the findings in LF patients, the severity of intranasal and oral routes in the mouse model was correlated with an increase in the frequency of circulating T cells with homing signatures for the respiratory mucosa (CCR3 and CCR4) and the gut (ITGA4B7) (Fig. 6F), which suggests that the identification of circulating T cells with defined homing markers during acute LF may provide useful information about routes of infection. These data, together with the observations in human LF cases, strongly suggest that mucosal exposure to LASV is associated with severe disease and high lethality.

Discussion
In this study we present clinical immunology data collected in LF patients treated at the ISTH Lassa ward during the Nigerian LF outbreak of 2018. There are several clades of LASV circulating in Nigeria, and clade distribution is closely related with geography. Since all patients came from Edo and its neighboring states, it is highly likely that all patients were infected with viruses of the lineage II (30). Still, a possible confounding factor in our study could be the association of specific viruses with pathogenesis, although this would be more likely at the sub-lineage level.

With this caveat in mind, our research was focused on trying to understand the role of T cells in LF pathogenesis. Previous work has highlighted the potential role of T-cell-mediated immunopathology of LASV infection in animal models (6, 7). However, there is much less information about whether T cell-mediated immunopathology may influence severe and fatal LF in humans.

We observed that, during the acute phase of LF, there was substantial activation of T cells that were antigenically-unrelated to LASV. In particular, we demonstrated that EBV-specific CD8 T cells were activated during acute LF in humans and that OVA-specific T cells were also activated during LASV infection in a mouse model. The first finding could be due to activation of bystander T cells and/or antigen-specific activation of CD8 T cells due to EBV reactivation. Reactivation of latent virus infections including EBV and CMV has been previously reported as a consequence of alterations of immune homeostasis leading to inflammation and immune suppression (14, 26), and has been observed in severe viral infections such as Ebola virus disease (14) and influenza (31). The OT-I T-cell activation observed in the mixed bone marrow chimera mouse model however, can only be due to bystander T-cell activation, since the stimulating antigen (OVA) was never present. This phenomenon is common to many viral infections or autoimmune disorders. In the context of viral infection, bystander T-cell activation may result in either enhanced immunity or enhanced immunopathology (26, 31, 32). Although our data are not
quantitative, there are some indications that non-specific T-cell activation may be an important component of LF immunopathology. We have observed that in a mouse model of infection, bystander T cells generated after LASV infection have the capacity to migrate to inflamed tissues. There is substantial evidence that recruitment of bystander T cells to mucosal tissues can cause tissue damage (15, 32, 33). Our findings in the mouse model are also in agreement with the tSNE analysis of T cells from human fatalities, which showed homing capacity of T cells with a central memory phenotype. In addition, we speculate that the activation of T cells antigenically-unrelated with LASV may be correlated with the hyperexpansion of T-cell clones during the early time points after disease onset in fatal LF. Since fatal LF cases sustained high levels of viremia throughout the course of disease, it is conceivable that a fatal outcome may be in part attributed to the expansion of non-specific T-cell clones that dominate the response and are unable to control virus replication. For example, activation of antigenically-unrelated T cells has been shown to delay recruitment of virus-specific T cells to the lungs in mouse models of respiratory virus infections (34), and bystander T cells formed during virus infection have been shown to enhance virus-induced autoimmune responses (35). An additional consequence of bystander T-cell formation, is conversion of effector CD4 T cells into Foxp3+ peripheral regulatory T cells (Tregs). This has been shown previously to promote a regulatory environment in which antigen-specific T-cell immune responses are diminished (19). In that regard, we have observed that LASV-specific effector T-cell responses are remarkably diminished in severe and fatal LF with a direct association between elevated viremia and serum aminotransferases and poor T-cell functionality. The poor LASV-specific T-cell effector responses observed in severe LF are also in agreement with previously published in vitro data in which direct infection of DCs led to poor T-cell activation (36). Although we do not know whether LASV primarily infects antigen-presenting cells in humans, we observed a
correlation between high viral loads and poor formation of LASV-specific effector T cells.

In a IFNAR\textsuperscript{B6} chimera with functional hematopoietic system, inoculation of LASV via different routes resulted in significantly different degrees of disease severity. This finding was in agreement with the association between severe LF and lymphocyte homing to gut and respiratory mucosa observed in humans. Interestingly, in these IFNAR chimera mice, mucosal exposure (oral and intranasal) resulted in remarkably higher lethality than skin exposure. Although we do not have exposure data in humans, previous studies have demonstrated that, as opposed to EVD, LF is caused by multiple spillover events from rodents into the human population, rather than by human-to-human transmission (5, 37). Epidemiological studies performed during previous LF outbreaks suggested that risk factors for infection with LASV include activities that may involve contact with rodent excreta, such as consumption of contaminated food or inhalation of dust particles harboring virus particles (3, 4, 38). Skin contact with rodents via bites or while preparing rodents for food have been also reported (2, 39). It would be worthwhile to further investigate whether mucosal exposure to LASV leads to a more severe phenotype than skin exposure. These experiments could also serve to determine whether lymphocyte homing signatures could provide information about routes of transmission and therefore disease severity, which has the potential for significant public health relevance.

Finally, our data suggest that, in severe LF, T-cell activation does not necessarily lead to efficient LASV-specific T-cell responses and virus control in humans, but rather results in enhanced immunopathology and disease severity. This is in agreement with previous findings in a LASV-susceptible mouse model (6). In addition, similar studies conducted in mice infected with lymphocytic choriomeningitis
virus (LCMV), the prototypic old-world arenavirus, have underscored a dual role of CD8 T cells during infection. Whereas, CD8 T cells are essentially required for early control of LCMV replication, they can also worsen immune-mediated pathology in more severe models of infection (40). These results are therefore in line with our findings in human LF. Although counterintuitive, we propose that it could be worthwhile to test the effect of post-exposure therapies aimed at depleting T cells in acute LF, at least in preclinical models such as NHPs and perhaps in combination with antivirals.

Methods

Ethics statement.
The present study was performed with approval of the Irrua Specialist Teaching Hospital (ISTH) Research and Ethics Committee (ISTH/HREC/20171208/43) and the Ethics Committee of the Medical Association of the State of Hamburg (PV3186).

Patients and samples
All samples were obtained in the period between January 2017 and June 2018 from acute LASV patients that were diagnosed and treated at Irrua Specialist Teaching Hospital in Edo state, Nigeria. Patients were enrolled in the study at admission to the Lassa ward at ISTH. Samples were collected from patients every two days from the day of admission and until they were discharged. All patients received Ribavirin treatment upon their admission to the ward. Lassa diagnostics and viral loads were determined in EDTA-whole blood samples using the commercial RT-PCR Lassa Kit 1.0 (Altona Diagnostics). For evaluation of clinical chemistry parameters including AST levels, plasma was analyzed using a SpotChem machine.

Isolation of PBMCs and HLA typing
Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using Lymphoprep gradient and SepMate technology. PBMCs were either directly used for analysis or cryopreserved in 90% heat inactivated fetal calf serum (FCS) and 10% dimethylsulfoxid (DMSO) for posterior analysis. For selection of HLA-A2+ patient samples DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer’s instructions. DNA was used for PCR based genotyping using an HLA-A low resolution screening kit (OLERUP).

Phenotyping of cells with flow cytometry

Cells were washed with phosphate-buffered saline (PBS) prior to staining. Cryopreserved human PBMCs were thawed in pre-warmed RPMI-1640 medium containing 10% human serum (HS), 1x Streptomycin, 1x Penicillin, 1x L-Glutamine and in the presence of DNAse I. Cell viability was determined using a live/dead dye staining for 20 minutes (1/1000 dilution) (Zombie dye, Biolegend). Fcγ receptor blocking was performed with either murine or human BD Fc Block (BD). After blocking, cells were stained with antibodies against extracellular antigens with an antibody cocktail diluted in PBS containing 5% fetal calf serum (FCS). After that, cells were fixed and permeabilized with Cytofix/Cytoperm (BD) for 45 minutes and then stained with antibodies against intracellular antigens if required (Table 1). Samples were either acquired using a bench top Guava (Millipore) (Nigeria) cytometer or an LSR Fortessa (BD) cytometer (Hamburg). Data analysis was done with FlowJo software (TreeStar). Cell population gating was adjusted with Fluorescence-minus-one (FMO) controls. Viability was assessed in relation to corresponding AST serum levels (U/L) by linear regression.

For identification of epitope-specific CD8 T cells we utilized staining with HLA-A*02:01-based tetramers conjugated with previously validated LASV and EBV peptides. Tetramers were generated using a Custom Flex-T™ MHC Tetramer kit
High-dimensional analysis of flow cytometry data

Multiparametric analysis of data was performed in R statistical software using gplots, mclust and factoextra packages. Clustering was performed by “ward.D2” and euclidian distance based algorithm. Principal component analysis was performed on correlation matrix. Cluster number was chosen manually based on hierarchical clustering of samples by hclust function.

For tSNE analyses the population of activated T cells (CD8 and CD4, seperately, were selected and concatenated across all samples for each disease category respectively using FlowJo software). These pools were then downsized based on least abundant population and again merged to generate one FSC file on which tSNE analysis was performed using the tSNE plugin. Populations were gated by automated density selection. Contribution of cells originating from the three outcome pools and expression frequency of homing markers were identified for each population.

TCR repertoire analysis

TCR sequences were obtained by amplifying the TCR β CDR3 loci from samples of PBMCs RNA. The TCR library was generated via Multiplex PCR using 45 forward primers each specific to a functional TCR Vβ segment and 13 reverse primers each specific to a TCR Jβ segment. Forward and reverse primers were pooled separately to a final stock of 10 μM each. Multiplex PCR amplification was performed in a first step with the use of a 1:5 mix of primers without and with overhang adapters, which serve as the specific target of primers in a second PCR amplification. The Qiagen multiplex PCR kit was used at a final volume of 25 μl including 1 μl of DNA template and 2.5 μl of Q solution. The PCR cycle was initiated by a 15 minutes step of
activation of the HotStartTaq DNA Polymerase at 95°C. Next, denaturation at 94°C for 30 seconds was followed by annealing for 90 seconds at 69°C, with touchdown PCR of 1°C reduction of the temperature for the first 10 cycles. After extension at 72°C for 90 seconds, 40 cycles (10 cycles for touchdown + 30 cycles) of denaturation, annealing, and extension followed. A final extension at 72°C was performed for 10 minutes. A second PCR step was performed to include Nextera XT dual indices as well as Illumina sequencing adapters. The Qiagen multiplex PCR was used once again in a final volume of 25 μl including 5 μl of DNA template and 2.5 μl of each primer. The PCR cycle was initiated with an activation step of 15 minutes at 95°C followed by 15 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C and extension for 30 seconds at 72°C. A final extension at 72°C was performed for 5 minutes. PCR products were purified after each PCR run with the use of Agencourt AMPure XP reagent and following the manufacturer’s instructions. PCR products were mixed with 1.8x volume of beads for the recovery of amplicons of about 200bp. The ‘R’ package tcR was used for TCR data analysis. Simpson inverse diversity was calculated using repDiversity function based on read proportion. Clonal expansion was calculated using clonal.space.homeostasis function defining clones as Rare (0 < X <= 1e-05), Small (1e-05 < X <= 1e-04), Medium (1e-04 < X <= 0.001), Large (0.001 < X <= 0.01) and Hyperexpanded (0.01 < X <= 1).

T-cell stimulation assays

PBMCs were resuspended in 10% HS, 1x Streptomycin, 1x Penicillin, 1x L-Glutamine at a concentration of 2.5x10^6 cells/ml. Cells were stimulated with a LASV-specific peptide pool at concentration of 1 μg/ml for each individual peptide. All peptides were selected using the immune epitope data base (IEDB) prediction tool utilizing an artificial neural network prediction method (ANN) for MHC-I binding prediction. Peptides were predicted for all common HLA-A and HLA-B alleles to cover >95% of the Nigerian population. Only peptides with a predicted IC_{50} < 100
nmol were selected. A peptide pool was generated comprising peptides found in both LASV strain Ba366 (GU830839.1), which is a commonly used lab strain, and a field isolated strain Nig-08-A47 (Irrua, Nigeria 2008) (ADU56631.1) to increase the likelihood of sequence conservation. PMA/ionomycin (50 ng/ml, 2.5 µM) was used as a positive stimulation control. PBMCs were incubated with the peptides for 6 hours (37°C, 5% CO₂). To prevent cytokine release into the medium, cells were treated with GolgiStop™ (BD) protein transport inhibitor (1/1000 dilution) for the last 5 hours. Plates were sealed airtight and placed at 4°C overnight, then stained for flow cytometric analysis. Viability was assessed in relation to corresponding AST serum levels (U/L) by linear regression. After background subtraction, negative values were set equal to 0, and all responses above 0 were considered for each cytokine separately. Responses were considered as multifunctional for the respective study sample, if response to peptide stimulus resulted in above-background responses for multiple cytokines within the respective CD8 T-cell population.

Mice

All mouse strains C57BL/6, C57BL/6.Ly5.1 (B6.SJL-Ptprc a Pebp/BoyJ), IFNAR⁻⁻ (B6.Cg-Ifnar1tm1.2Ees/J) and OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J) were purchased from The Jackson Laboratory. All mouse colonies were maintained in the animal housing facility at BNITM, Hamburg. All animal experiments were conducted according to the guidelines of the German animal protection law and under approvals 31/17 and 92/18 issued by the state of Hamburg. Bone marrow chimeras were generated as previously described (41). Briefly, 6-10-week-old recipient mice were lethally irradiated (550 rad, 4 h apart by a cesium source) and reconstituted with $3 \times 10^6$ donor bone marrow cells.

Experimental infection of mice
Mice were infected with 1000 FFU of LASV (recombinant Ba36 strain). Intranasal inoculation was performed under isoflurane anesthesia by application of 25 µl of virus diluted in PBS directly to the nostrils. Oral administration of virus was performed via application of 100 µL inoculum with the aid of a sterile buttoned cannula (22G), 3 cm in length and 1.25 mm in diameter. Intradermal inoculation of LASV was also performed under isoflurane anesthesia. Using an electric shaver, fur was shaved off in a 1x1 cm area on the back of the mouse, just above the tail base. Hairs were brushed aside, and the skin was gently scratched using UV sterilized sandpaper (P150 to P400 granularity). On the scratched surface 10 µl sterile inoculum was pipetted and distributed evenly. After application mice were kept under anesthesia for 15 minutes.

Animals with severe signs of disease such as temperature < 28°C or weight loss > 20% were euthanized as per our approved protocol guidelines. For evaluation of clinical chemistry and viremia, 30–50 µl of blood was drawn by tail vein puncture every 3–7 days over the course of the experiment. Levels of AST in serum were analyzed with a commercial kit and using a Fujifilm machine. Sera were diluted 1:10 or higher with a 0.9% saline solution (v/v). The normal range of AST in bone marrow chimeric mice has been determined as < 200 U/l. Viremia and virus titers in organs were determined using plaque assays to determine the presence of focus-forming units as described elsewhere. When criteria for euthanasia were fulfilled or at the end of the experiment, animals were euthanized with an isoflurane overdose followed by cervical dislocation. Murine PBMCs were isolated from whole blood samples collected at day 9 post-infection. Red blood cell lysis was performed using Red Blood Cell Lysis Buffer (10x; Biolegend) according to the manufacturer’s instructions. PBMCs were directly analyzed through flow cytometry.
All staff carrying out animal experiments passed training programs according to category B or C of the Federation of European Laboratory Animal Science Associations.

Acknowledgements

This work was funded by the German Science Foundation (DFG) through its program for cooperation with Africa (DFG-Infectiology grant MU 3565/3-0 to CMF and GU 883/5-1 to SG). This work was also partially supported by the German Center for Infection Research (DZIF) grant TTU 01.702 to SG and CMF.

References

1. McCormick JB, Webb PA, Krebs JW, Johnson KM, Smith ES. 1987. A prospective study of the epidemiology and ecology of Lassa fever. J Infect Dis 155:437–444.

2. Lecompte E, Fichet-Calvet E, Daffis S, Koulemou K, Sylla O, Kourouma F, Doré A, Soropogui B, Aniskin V, Allali B, Kouassi Kan S, Lalib A, Koivogui L, Günther S, Denys C, Meulen ter J. 2006. Mastomys natalensis and Lassa fever, West Africa. Emerging Infect Dis 12:1971–1974.

3. Ogbu O, Ajuluchukwu E, Uneke CJ. 2007. Lassa fever in West African sub-region: an overview. J Vector Borne Dis 44:1–11.

4. Ajayi NA, Nwigo CG, Azuogu BN, Onyire BN, Onwenu EU, Ogbonnaya LU, Onwe FI, Ekaete T, Günther S, Ukwaja KN. 2013. Containing a Lassa fever epidemic in a resource-limited setting: outbreak description and lessons learned from Abakaliki, Nigeria (January-March 2012). Int J Infect Dis 17:e1011–6.

5. Kafetzopoulou LE, Pullan ST, Lemey P, Suchard MA, Ehichioya DU, Pahlmann M, Thielebein A, Hinzmarn J, Oestereich L, Wozniak DM, Efthymiadis K, Schachten D, Koenig F, Matjeschek J, Lorenzen S, Lumley S, Ighodalo Y, Adomeh DI, Olokoh T, Omonoh E, Omiunu R, Agbukor J, Ebo B, Aiyepada J, Ebhodaghe P, Osieni B, Ehikhametalor S, Akhilemen P, Airende M, Esumeh R, Muoebonam E, Giwa R, Ekanam A, Igenegbale G, Odigie G, Okonofua G, Enigbe R, Oyakhilome J, Yerumoh EO, Ocia I, Aire C, Okonofua M, Atafo R, Tobin E, Asogun D, Akpede N, Okokhere PO, Rafiu MO, Iraoyah KO, Iruolaghe CO, Akhideno P, Erameh C, Akpede G, Isibor E, Naidoo D, Hewson R, Hiscox JA, Vipond R, Carroll MW, Ihekweazu C, Formenty P, Okogbenin S, Ogbona-Emovon E, Günther S.
643 Duraffour S. 2019. Metagenomic sequencing at the epicenter of the Nigeria
644 2018 Lassa fever outbreak. Science 363:74–77.
645
646 6. Oestereich L, Lüdtke A, Ruibal P, Pallasch E, Kerber R, Rieger T, Wurr S,
647 Bockholt S, Pérez-Girón JV, Krassmann S, Günther S, Munoz-Fontela C.
648 2016. Chimeric Mice with Competent Hematopoietic Immunity Reproduce Key
649 Features of Severe Lassa Fever. PLoS Pathog 12:e1005656.
650
651 7. Flatz L, Rieger T, Merkler D, Berghaler A, Regen T, Schedensack M,
652 Bestmann L, Verschmidt M, Brück W, Hanisch U-K, Günther
653 S, Pinschewer DD. 2010. T cell-dependence of Lassa fever pathogenesis.
654 PLoS Pathog 6:e1000836.
655
656 8. McElroy AK, Akondy RS, Harmon JR, Ellebedy AH, Cannon D, Klena JD,
657 Sidney J, Sette A, Mehta AK, Kraft CS, Lyon MG, Varkey JB, Ribner BS,
658 Nichol ST, Spiropoulou CF. 2017. A Case of Human Lassa Virus Infection
659 With Robust Acute T-Cell Activation and Long-Term Virus-Specific T-Cell
660 Responses. Journal of Infectious Diseases 215:1862–1872.
661
662 9. Baize S, Marianneau P, Loth P, Reynard S, Journeaux A, Chevallier M,
663 Tordo N, Deubel V, Contamin H. 2009. Early and strong immune responses
664 are associated with control of viral replication and recovery in Lassa virus-
665 infected cynomolgus monkeys. J Virol 83: 5890-5903
666
667 10. Fisher-Hoch S, McCormick JB, Sasso D, Craven RB. 1988. Hematologic
dysfunction in Lassa fever. J Med Virol 26:127–135.
668
669 11. Monjazeb AM, Tietze JK, Grossenbacher SK, Hsiao H-H, Zamora AE,
670 Mirsoian A, Koehn B, Blazar BR, Weiss JM, Wiltrout RH, Sckisel GD,
671 Murphy WJ. 2014. Bystander activation and anti-tumor effects of CD8+ T cells
672 following Interleukin-2 based immunotherapy is independent of CD4+ T cell
673 help. PLoS ONE 9:e102709.
674
675 12. Zhang N, Bevan MJ. 2011. CD8(+) T cells: foot soldiers of the immune
676 system. Immunity 35:161–168.
677
678 13. Whiteside SK, Snook JP, Williams MA, Weis JJ. 2018. Bystander T Cells: A
679 Balancing Act of Friends and Foes. Trends Immunol 39:1021–1035.
680
681 14. Agrati C, Castilletti C, Casetti R, Sacchi A, Falasca L, Turchi F, Tumino N,
682 Bordoni V, Cimini E, Viole D, Lalle E, Borti L, Lanini S, Martini F, Nicastri
683 E, Petrosillo N, Puro V, Piacentini M, Di Cara A, Kobinger GP, Zumla A,
684 Ippolito G, Capobianchi MR. 2016. Longitudinal characterization of
dysfunctional T cell-activation during human acute Ebola infection. Cell Death
685 Dis 7:e2164–e2164.
686
687 15. Maurice NJ, McElrath MJ, Andersen-Nissen E, Frahm N, Prlic M. 2019.
688 CXCR3 enables recruitment and site-specific bystander activation of memory
689 CD8+ T cells. Nat Commun 10:4987–15.
690
691 16. Rivino L, Kumaran EA, Thein T-L, Too CT, Gan VCH, Hanson BJ, Wilder-
692 Smith A, Bertoletti A, Gascoigne NRJ, Lye DC, Leo YS, Akbar AN,
693 Kemeny DM, Macary PA. 2015. Virus-specific T lymphocytes home to the
694 skin during natural dengue infection. Sci Transl Med 7:278ra35–278ra35.
17. Koelle DM, Liu Z, McClurkan CM, Topp MS, Riddell SR, Pamer EG, Johnson AS, Wald A, Corey L. 2002. Expression of cutaneous lymphocyte-associated antigen by CD8(+) T cells specific for a skin-tropic virus. J Clin Invest 110:537–548.

18. Shannon B, Yi TJ, Thomas-Pavanel J, Chieza L, Janakiram P, Saunders M, Tharao W, Huibner S, Remis R, Rebbapragada A, Kaul R. 2014. Impact of asymptomatic herpes simplex virus type 2 infection on mucosal homing and immune cell subsets in the blood and female genital tract. J Immunol 192:5074–5082.

19. Thompson LJ, Lai J-F, Valladao AC, Thelen TD, Urry ZL, Ziegler SF. 2016. Conditioning of naive CD4(+) T cells for enhanced peripheral Foxp3 induction by nonspecific bystander inflammation. Nat Immunol 17:297–303.

20. Woodland DL, Kohlmeier JE. 2009. Migration, maintenance and recall of memory T cells in peripheral tissues. Nat Rev Immunol, 2009 ed. 9:153–161.

21. Rott LS, Rosé JR, Bass D, Williams MB, Greenberg HB, Butcher EC. 1997. Expression of mucosal homing receptor alpha4beta7 by circulating CD4+ cells with memory for intestinal rotavirus. J Clin Invest 100:1204–1208.

22. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song S-Y. 2004. Retinoic acid imprints gut-homing specificity on T cells. Immunity 21:527–538.

23. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, Andrian Von UH. 2003. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 424:88–93.

24. McElroy AK, Akondy RS, Davis CW, Ellebedy AH, Mehta AK, Kraft CS, Lyon GM, Ribner BS, Varkey J, Sidney J, Sette A, Campbell S, Ströher U, Damon I, Nichol ST, Spiropoulou CF, Ahmed R. Human Ebola virus infection results in substantial immune activation. Proc Natl Acad Sci USA 112:4719-4724.

25. Ruibal P, Oestereich L, Lüdtke A, Becker-Ziaja B, Wozniak DM, Kerber R, Korva M, Cabeza-Cabreroiz M, Bore JA, Koundouno FR, Duraffour S, Woller R, Thorenz A, Cimini E, Viola D, Agrati C, Repits J, Afrough B, Cowley LA, Ngabo D, Hinzmann J, Mertens M, Vitoriano I, Logue CH, Boettcher JP, Pallasch E, Sachse A, Bah A, Nitzsche K, Kuisma E, Michel J, Holm T, Zekeng E-G, Garcia-Dorival I, Wölfl R, Stoeker K, Fleischmann E, Strecker T, Di Caro A, Avšič-Županc T, Kurth A, Meschi S, Mély S, Newman E, Bocquin A, Kis Z, Kelterbaum A, Molkenthin P, Carletti F, Portmann J, Wolff S, Castilletti C, Schudt G, Fizet A, Ottowell LJ, Herker E, Jacobs T, Kretschmer B, Severi E, Ouedraogo N, Lago M, Negredo A, Franco L, Anda P, Schmiedel S, Kreuels B, Wichmann D, Addo MM, Lohse AW, de Clerck H, Nancares C, Jonckheere S, Van Herp M, Sprecher A, Xiaojiang G, Carrington M, Miranda O, Castro CM, Gabriel M, Drury P, Formenty P, Díaz-Ridaura L, Koivogui L, Magassouba N, Carroll MW, Günter S, Munoz-Fontela C. 2016. Unique human immune signature of Ebola virus disease in Guinea. Nature 533:100–104.

26. Doisne J-M, Urrutia A, Lacabaratz-Porret C, Goujard C, Meyer L, Chaix M-L, Sinet M, Venet A. 2004. CD8+ T cells specific for EBV, cytomegalovirus,
and influenza virus are activated during primary HIV infection. J Immunol 173:2410–2418.

27. Danilova E, Skrindo I, Gran E, Hales BJ, Smith WA, Jahnsen J, Johansen FE, Jahnsen FL, Baekkevold ES. 2015. A role for CCL28-CCR3 in T-cell homing to the human upper airway mucosa. Mucosal Immunol 8:107–114.

28. Mikhak Z, Strassner JP, Luster AD. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. J Exp Med 210: 1855-1869.

29. Takamura S, Roberts AD, Jelley-Gibbs DM, Wittmer ST, Kohlmeier JE, Woodland DL. 2010. The route of priming influences the ability of respiratory virus-specific memory CD8+ T cells to be activated by residual antigen. J Exp Med 207:1153–1160.

30. Ehichioya DU, Dellicour S, Pahlinmann M, Rieger T, Oestereich L, Becker-Ziaja B, Cedar D, Ighodalo Y, Olokor T, Omomoh E, Oyakhilome J, Omiunu R, Agbukor J, Ebo B, Aiyepada J, Ebhodaghe P, Osiemi B, Ehikhametalor S, Akhilomen P, Aire C, Akpede N, Tobin E, Ogbaire-Evan E, Okunofua G, Enigbe R, Omonogho Yerumoh E, Palla E, Boekholt S, Kaufzopoulou LE, Duraffour S, Okohere PO, Akpede GO, Okogbenin SA, Odia I, Aire C, Akpede N, Tobin E, Ogbaire-Evan E, Lemey P, Adomeh DI, Asogun DA, Günther S. 2019. Phylogeography of Lassa Virus in Nigeria. J. Virol 93:684.

31. Furman D, Joci J, Sharma S, Shen-Orr SS, Angel CJL, Onengut-Gumuscu S, Kidd BA, Maecker HT, Concannon P, Dekker CL, Thomas PG, Davis MM. 2015. Cytomegalovirus infection enhances the immune response to influenza. Sci Transl Med 7:281ra43–281ra43.

32. Crosby EJ, Goldschmidt MH, Wherry EJ, Scott P. 2014. Engagement of NKG2D on Bystander Memory CD8 T Cells Promotes Increased Immunopathology following Leishmania major Infection. PLoS Pathog 10:e1003970.

33. Ghani S, Feuerer M, Doebis C, Lauer U, Loddenkemper C, Huen H, Hamann A, Syrbe U. 2009. T cells as pioneers: antigen-specific T cells condition inflamed sites for high-rate antigen-non-specific effector cell recruitment. Immunology 128:e870–80.

34. Ostler T, Pircher H, Ehl S. 2003. “Bystander” recruitment of systemic memory T cells delays the immune response to respiratory virus infection. Eur J Immunol 33:1839–1848.

35. Chen AM, Khanna N, Stolnman SA, Bergmann CC. 2005. Virus-specific and bystander CD8 T cells recruited during virus-induced encephalomyelitis. Journal of Virology 79:4700–4708.

36. Schaeffer J, Carnec X, Reynard S, Mateo M, Picard C, Pietrosemoli N, Dillies M-A, Baize S. 2018. Lassa virus activates myeloid dendritic cells but suppresses their ability to stimulate T cells. PLoS Pathog 14:e1007430.
37. Andersen KG, Shapiro BJ, Matranga CB, Sealfon R, Lin AE, Moses LM, Folarin OA, Goba A, Odia I, Ehiane PE, Momoh M, England EM, Winnicki S, Branco LM, Gire SK, Phelan E, Tariyal R, Tewhey R, Omoniwa O, Fullah M, Fonnie R, Fonnie M, Kanneh L, Jalloh S, Gbakie M, Saffa S, Karbo K, Gladden AD, Qu J, Stremlau M, Nekoui M, Finucane HK, Tabrizi S, Vitti JJ, Birren B, Fitzgerald M, McCowen C, Ireland A, Berlin AM, Bochicchio J, Tazon-Vega B, Lennon NJ, Ryan EM, Bjornson Z, Milner DA, Lukens AK, Brodie N, Rowland M, Heinrich M, Akdag M, Schieffelin JS, Levy D, Akpan H, Bausch DG, Rubins K, McCormick JB, Lander ES, Günter S, Hensley L, Okogbenin S, Viral Hemorrhagic Fever Consortium, Schaffner SF, Okohere PO, Khan SH, Grant DS, Akpede GO, Asogun DA, Gnirke A, Levin JZ, Happi CT, Garry RF, Sabeti PC. 2015. Clinical Sequencing Uncovers Origins and Evolution of Lassa Virus. Cell 162:738–750.

38. Tobin. 2015. Lassa fever in Nigeria: Insights into seroprevalence and risk factors in rural Edo State: A pilot study. Journal of Medicine in the Tropics 17:51.

39. Kerber R, Reindl S, Romanowski V, Gómez RM, Ogbaini-Emovon E, Günter S, Meulen ter J. 2015. Research efforts to control highly pathogenic arenaviruses: a summary of the progress and gaps. J Clin Virol 64:120–127.

40. Baccala R, Welch MJ, Gonzalez-Quintial R, Walsh KB, Teijaro JR, Nguyen A, Ng CT, Sullivan BM, Zarpellon A, Ruggeri ZM, de la Torre JC, Theofilopoulos AN, Oldstone MBA. 2014. Type I interferon is a therapeutic target for virus-induced lethal vascular damage. Proc Natl Acad Sci USA 111:8925–8930.

41. Pérez-Girón JV, Belicha-Villanueva A, Hassan E, Gómez-Medina S, Cruz JLG, Lüdtke A, Ruibal P, Albrecht RA, García-Sastre A, Munoz-Fontela C. 2014. Mucosal Polyinosinic-Polycytidylic Acid Improves Protection Elicited by Replicating Influenza Vaccines via Enhanced Dendritic Cell Function and T Cell Immunity. J Immunol 193:1324–1332.

Figure legends

Figure 1. Patient demographics and clinical symptoms.

(A) Outcome (n=214), (B) gender (n=129) and age (n=63) distribution of patients admitted to the Lassa ward during the study period. Distribution of patients population is shown as the percentage of total cohort (%). Pie chart colors refer to legends below. (D) Symptoms of patients (n=136) admitted to the Lassa ward. (E) PBMCs were analysed for 36 patients in whole-blood samples in the field for
phenotypical assessment, for 13 patients for assessment of the T-cell response and for 22 acute patients cryopreserved samples were analyzed phenotypically. The graph depicts patients and date of sample collection according to the self-reported day post-onset (DPO). (F) DPO of patients at the time of sample collection was compared between patient groups with different outcomes. Violin plots depict individual samples, sample distribution, median and quantiles. ns=not-significant (Kruskal-Wallis, followed by Dunn’s). Abbreviations: ISTH, Irrua Specialist Teaching Hospital; DPO, days post-onset; NA, notanswered. (G) Viability of all fresh and frozen PBMC samples was assessed in relation to the corresponding AST serum levels (U/L). Panels depicts individual samples (fresh and frozen) and linear regression line with 95% confidence intervals (dashed).

Figure 2. T-cell activation during acute LF.
(A) HLA-A2 tetramers labeled with two fluorophores (T1 and T2, respectively) were utilized to track epitope-specific CD8 T cells during acute LF. The plots show pooled (concatenated) samples from LF patients (n=3) collected during the acute phase of LF or after discharge. (B) Overlay plots showing LASV epitope-specific CD8 T cells (red) plotted over the total activated (CD38+ HLA-DR+) population. (C) Epstein-Barr virus (EBV)-specific CD8 T cells were identified in acute LF patients through the use of tetramers labeled with two fluorophores (T1 EBV and T2 EBV). Plots show tetramer staining in negative control (LASV A2+) and uninfected control (LASV A2`). and a representative acute HLA-A2+ LF patient (n=2) (LASV+ A2`). Bottom plots show overlay of EBV-specific CD8 T cells over total activated CD8 T cells. (D) Mixed CD45.1 (WT) and OT-I bone marrow chimeras were generated and infected intranasally with 1000 FFU LASV as depicted in the schematic. (E) Frequency (%) of effector cells (CD44+ CD62L) is shown for CD8 T cells isolated from mouse PBMCs 9 days after infection (LASV, white) as compared to uninfected mice (control, black).
Figure 3. Clonal repertoires and effector T-cell responses in LF patients.

(A) Cross-sectional T-cell repertoire diversity in LF survivors (n=28) and fatalities (n=10) was analyzed by TCR sequencing and is represented as the inverse of Simpson's Index. Violin plots depict individual samples, sample distribution, median and quartiles. Statistical significance was determined using Mann-Whitney test and significance levels are presented as ns = p > 0.05; * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001; **** = p ≤ 0.0001. (B) Longitudinal evaluation of hyperexpanded clones (see clonal space definitions in Methods section) in n=120 samples collected from survivors and n=22 samples collected from non-surviving patients. Individual samples and trend lines are depicted. ns=not significant (Spearman’s correlation analysis). (C) Pie chart depicting the percentage of patient samples (n=26) that showed effector response capacity as indicated by cytokine production IFN-γ, TNF-α and degranulation CD107a/LAMP-1 in response to peptide stimulation. (D) Graphs representing cytokine responses of CD8 T cells in LF patients stimulated with LASV peptide pools. Cytokine responses and degranulation are reported across ranges of patient levels of serum AST (U/L) (n=21). Violin plots depict individual samples, samples distribution, median and quantiles. ns=not-significant (Kruskal-Wallis, followed by Dunn’s). (E) Cytokine responses and degranulation are reported across cycle threshold (Ct) values as a readout of patient viremia (n=20). Individual samples
are depicted. Abbreviations: AST, aspartate aminotransferase; ct, cycle threshold; DPO, days post-onset; IFN, interferon; LASV, Lassa virus, TNF, tumor necrosis factor.

Figure 4. T-cell homing in human LF.
(A) Freshly isolated patient PBMCs were analyzed by flow cytometry and activated (CD38$^+$ HLA-DR$^+$) CD8 T cells from LF patients were analyzed for expression of the T-cell homing factors ITGB7, ITGA4, ITGB1 (n=25), CCR3 (n=16), and CLA (n=61). Violin plots depict individual samples, samples distribution, median and quantiles. Statistical significance was determined using Kruskal-Wallis test, followed by Dunn's post-test and significance levels are presented as ns = p > 0.05; * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001; **** = p ≤ 0.0001. (B) Principal component analysis (PCA) of T-cell homing markers in the activated compartment (CD38$^+$ HLA-DR$^+$) of CD4 and CD8 T cells in cryopreserved patient samples (n=54). Clustering of samples was based on hierarchical analysis to identify main subsets. (C) CFR for each cluster was calculated based on outcome of disease of the corresponding patient linked to each sample. Abbreviations: Dim, dimension; C, cluster; CFR, case fatality ratio.

Figure 5. T-cell populations in LF patients.
(A) Activated (CD38$^+$ HLA-DR$^+$) CD8$^+$ and CD4$^+$ T cells were concatenated across all patient samples (n=54). tSNE was calculated on homing marker expression of CLA, CCR3, CCR4, CCR7, ITGA4, ITGA1, ITGA6 and ITGB7 and expression of CD45RA. Outcome-based population analysis was performed by automated density-based gating. (B) For each identified population, percentage (%) of contribution from each outcome was verified and expression frequency (%) of each marker is shown according to color bar on the right. Abbreviations: CCR, chemokine receptor; CLA, cutaneous lymphocyte antigen; ITG, integrin; tSNE, t-distributed stochastic neighboring embedding.
Figure 6. Route-dependent LF severity in mice.

(A) IFNAR<sup>B6</sup> chimeras were infected with 1000 FFU of LASV either i.n., p.o. or i.d. Uninfected mice served as control. Survival is shown in Kaplan-Meier curves. Statistical evaluation was performed via Mantel-Cox test. (B-E) Longitudinal analysis of morbidity parameters in IFNAR<sup>B6</sup> chimeras infected with LASV via different routes including relative weight loss (B), fluctuations in body temperature (C), levels of serum AST (D) and viremia (E). (F) Frequency of homing markers in peripheral blood cells was analyzed for each infection route 9 days post-infection by flow cytometry on effector (CD44<sup>+</sup> CD62L<sup>-</sup>) CD4 and CD8 T cells. In B-F, statistical significance was determined using two-way ANOVA, followed by Dunnett's multiple comparison test.

Across the figure, significance levels are presented as follows: ns = p > 0.05; * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001; **** = p ≤ 0.0001. In C, shaded areas highlight body temperature extremes due to fever (red > 37.4°C, blue < 33°C). In D-E, shaded area (grey) marks limit of detection. Abbreviations: AST, aspartate aminotransaminase; DOI, days post-infection; FFU, focus forming units; i.n., intranasally; i.d., intradermally; p.o., orally.
Table 1. Antibodies utilized for flow cytometry. The following table depicts all antibodies utilized in flow cytometry for characterization of T-cell phenotype and functional profile.

| REACTIVITY | ANTIBODY | CONJUGATED FLUOROPHORE | CLONE | COMPANY        |
|------------|----------|-------------------------|-------|----------------|
| α-human    | Integri α 1 /CD49a | APC | TS2/7 | Biolegend |
| α-human    | Integri α 4 /CD49d | PE/Dazzle 594 | 9F10  | Biolegend |
| α-human    | Integri α 4 /CD49d | APC | 9F10  | Biolegend |
| α-human    | Integri α E /CD103 | BV711 | Ber-ACT8 | BD Biosciences |
| α-human    | Integri α E /CD103 | BUV395 | Ber-ACT8 | BD Biosciences |
| α-mouse    | CD103    | PerCP/Cy5.5 | 2E7  | Biolegend |
| α-human    | CD14     | APC/Cy7 | HCD14 | Biolegend |
| α-human    | CD16     | APC/Cy7 | 3G  | Biolegend |
| α-human    | CD19     | Alexa Fluor 700 | HIB19 | Biolegend |
| α-human    | CD193 (CCR3) | APC/Cy7 | Clone5E8 | Biolegend |
| α-human    | CD193 (CCR3) | PerCP/Cy5.5 | Clone5E8 | Biolegend |
| α-human    | CD193 (CCR3) | PE | Clone5E8 | Biolegend |
| α-mouse    | CD193 (CCR3) | APC Fire™ 750 | J073E5 | Biolegend |
| α-human    | CD194 (CCR4) | BV605 | L291H4 | Biolegend |
| α-mouse    | CD194(CCR4) | PE/Cy7 | 2G12 | Biolegend |
| α-human    | CD195(CCR5) | BV421 | HEK/1/85a | Biolegend |
| α-human    | CD197(CCR7) | BV421 | G043H7 | Biolegend |
| α-human    | CD197(CCR7) | BV711 | G043H7 | Biolegend |
| α-human    | CD3      | BV421 | OKT3 | Biolegend |
| α-human    | CD3      | BV510 | OKT3 | Biolegend |
| α-mouse    | CD3      | FITC | 17A2 | Biolegend |
| α-human    | CD38     | PerCP/Cy5.5 | HIT2 | Biolegend |
| α-human    | CD38     | BV510 | HIT2 | Biolegend |
| α-mouse    | CD3ε     | PE/Dazzle 594 | 145-2C11 | Biolegend |
| α-human    | CD4      | PerCP/CY5.5 | SK3  | BD Biosciences |
| α-human    | CD4      | BUV737 | SK3  | BD Biosciences |
| α-human    | CD4      | BV711 | SK3  | Biolegend |
| Antibody         | Target         | Color       | Clone       | Company        |
|------------------|----------------|-------------|-------------|----------------|
| α-mouse CD4      | BUV737         | GK1.5       | BD Biosciences |
| α-mouse CD44     | BV395          | IM7         | BD Biosciences |
| α-mouse CD45.2   | PE             | 104         | eBioscience  |
| α-mouse CD45.1   | APC            | A20         | Biolegend    |
| α-mouse CD45.2   | FITC           | A20         | Biolegend    |
| α-mouse CD56     | BV711          | HI100       | Biolegend    |
| α-mouse CD56     | BV510          | HCD56       | Biolegend    |
| α-human CD56 (NCAM) | Alexa Fluor 700 | 5.1H11 | Biolegend |
| α-human CD62L    | BV785          | MEL-14      | Biolegend    |
| α-human CD8a     | FITC           | RPA-T8      | Biolegend    |
| α-human CD8a     | BV510          | RPA-T8      | Biolegend    |
| α-human CD8a     | PE/CY7         | RPA-T8      | Biolegend    |
| α-human CD8a     | BV650          | RPA-T8      | Biolegend    |
| α-mouse CD8a     | BV650          | 53-6.7      | Biolegend    |
| α-human/mouse CLA| PE             | HECA-452    | BD Pharmingen |
| α-human HLA-DR   | PE/CY7         | L243        | Biolegend    |
| α-human HLA-DR   | PerCP/Cy5.5    | L243        | Biolegend    |
| α-human HLA-DR   | FITC           | L243        | Biolegend    |
| α-human IFN-γ    | APC            | 4S.B3       | Biolegend    |
| α-human Integrin β 1 / CD29 | PE/CY7 | TS2/16 | Biolegend |
| α-human Integrin β 1 / CD29 | Alexa Fluor 700 | TS2/16 | Biolegend |
| α-human/mouse Integrin β 7 | PE | FIB27 | Biolegend |
| α-human/mouse Integrin β 7 | PerCP/Cy5.5 | FIB27 | Biolegend |
| α-human LAMP-1/α4β7 Integrin | PE/CY7 | H4A3 | Biolegend |
| α-mouse LPAM-1/α4β7 Integrin | BV421 | DATK32 | BD Biosciences |
| α-human TNF-α    | BV785          | MAb11       | Biolegend    |
| α-human TNF-α    | PE             | MAb11       | Biolegend    |
Port et al. Figure 4
