Hydrodynamic immunization leads to poor CD8 T-cell expansion, low frequency of memory CTLs and ineffective antiviral protection

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INTRODUCTION

A key feature of adaptive immunity underpinning the basis of immunization is the ability of the immune system to mount a specific response against a particular pathogen’s antigens (Ags), leading to induction of long-lived memory cells that are capable of immune clearance and protection upon reinfection. In general, naive adaptive immune cells are activated when they meet a professional Ag-presenting cell bearing their specific Ag in secondary lymphoid organs. Secondary lymphoid organs have highly organized structures allowing for the concentration of lymphocytes, which in turn enhances the access of Ag-presenting cells to specialized B and T cells for the initiation of adaptive immunity, involving activation, proliferation and expansion, as well as the subsequent development of protective effector cells. The activation of lymphoid-resident naive T cells by Ag-presenting cell grants the T-cell the ability to deploy to non-lymphoid tissues the Ag encoded by a DNA vaccine is delivered in a highly controlled fashion. We used hydrodynamic tail vein injection of plasmid to establish liver-specific LCMV-gp antigen (Ag) transient expression, and studied CD8 T cells induced using the P14 transgenic mouse model. CD8 T cells from this group exhibited unique and limited expansion, memory differentiation, polyfunctionality and cytotoxicity compared with T cells generated in intramuscularly immunized mice. This difference in liver-generated expansion resulted in lower memory CD8 T-cell frequency, leading to reduced protection against lethal viral challenge. These data show an unusual induction of naive CD8 T cells contributed to the lower frequency of Ag-specific CTLs observed after immunization in the liver, suggesting that limited priming in liver compared with peripheral tissues is responsible for this outcome.

Keywords: DNA vaccine; intrahepatic; intramuscular; immunization; CD8 T cells
Intravenous immunization was done by either directly injecting 200 µg of plasmid in 2 ml (about 10% volume of the mouse weight) of PBS solution within 7 to transiently transfect the LIV. Intramuscular (I.M.) immunization was done by either directly injecting 200 µg or electro-porating 20 µg of plasmid in 30 µl water into the tibialis anterior of P14 chimeras.

DNA plasmid vector
The plasmid construct was a gift from Dr Rafi Ahmed of Emory University, Atlanta, GA, USA. It contains the full-length LCMV-GP with five mutations. Only two of the mutations are in a specific epitope sequence. One of these two mutation changes the ending of the GP33 epitope ending by replacing methionine with cysteine.

GP33: KAVYNFATC
GP92: CSANNSHHY

Immunization
For hydrodynamic tail vein immunization (HDTV), mice were injected i.v. with 200 µg of plasmid in 30% sucrose for 24 h, frozen in OCT (frozen tissue matrix) and cut on a cryostat. The sectioned tissues of LIV were stained and images were obtained using a Zeiss Axiovert 100 inverted confocal microscope (Carl Zeiss, Thornwood, NY, USA). Analysis and quantification of fluorescence intensities were conducted using Image J software (National Institute of Health, Rockville, MD, USA).

Histology
For histology, LIVs were perfused with PBS and fixed for 24 h in 4% paraformaldehyde. These were then placed in 30% sucrose for 24 h, frozen in OCT (frozen tissue matrix) and cut on a cryostat. The sectioned tissues of LIV were stained and images were obtained using a Zeiss Axiovert 100 inverted confocal microscope (Carl Zeiss, Thornwood, NY, USA). Analysis and quantification of fluorescence intensities were conducted using Image J software (National Institute of Health, Rockville, MD, USA).

Antibodies, flow cytometry and intracellular cytokine staining
All antibodies were purchased from BD Biosciences (San Jose, CA, USA) except antibodies to mouse CD127, KLGM1 and PD-1, which were purchased from eBiosciences (San Diego, CA, USA). Major histocompatibility complex class I peptide tetramer to LCMV-gp33 was a gift from Dr Rafi Ahmed’s lab. Cells were stained for surface or intracellular proteins as described previously.12,18 Briefly, for intracellular cytokine staining, lymphocytes (10⁵ per well) were stimulated with the indicated peptides (0.2 µg ml⁻¹) in the presence of Brefeldin A and CD107a for 5 h followed by surface staining for CD8 and intracellular staining for interferon (IFN-)γ, tumor necrosis factor (TNF)-α or interleukin (IL)-2. The percent killing was calculated as follows: 100 – (% irrelevant peptide pulsed in infected)/(% irrelevant peptide pulsed in infected) × 100.

RESULTS
HI of LCMV-gp plasmid established acute LIV infection
HI of Ag-encoded DNA has been used previously as a model for acute HBV and HCV infection.22 Depending on the type of DNA vector or promoter,23 expression of the transgene can be transient or persistent. We used this model for expression of an LCMV-gp in mouse LIV (Figure 1a) after hydrodynamically injecting a CMV promoter-based plasmid DNA vector. Expression of this transgene product was exclusive to the LIV and absent in other major tissues (brain, heart, intestine, kidney, lymph nodes, lung (LUN), muscle, spleen (SPL) and skin). The expression of LCMV-gp was detectable at 16 h post injection and was largely lost by day 5 (Figure 1b).

Intrahepatic immunization induces equivalent activation markers and effector activities
Activation of CD8 T cells from both I.H. and I.M. immunized animals was determined by their levels of proliferation and effector phenotypic marker expressions. Five days following immunization, Ag-specific CD8 T cells were detectable as observed using D²GP33 tetramer to stain the PMBCs of these mice, indicating successful priming of naive CD8 T cells in both immunization groups. CFSE-labeled adoptively transferred T cells from the I.H. model exhibited quicker homeostatic proliferation during the first week of immunization in peripheral lymph node (PLN) and splenic lymph node (SPL) compared with the I.M. model (Figure 2a). Interestingly, proliferation in non-lymphoid tissues such as liver and lung at day 7 was, however, comparable between the two groups. Ag-specific CD8 T-cell proliferation in all tissues averaged around 98%, 2 weeks post immunization.

Next, we examined the expression levels of different activation markers, such as CD25 and CD44, and CD62L following CD8 T-cell priming in both models. We observed a significant increase in CD25 and CD44 expression on Thy1.1 CD8 T cells from both I.H. and I.M. mice in all tissues at day 7 and 14 post infection (Figure 2b). In addition, downregulation of CD62L, which is usually seen in effector CD8 T cells, was visible 14 days post infection. The comparable proliferation and effector phenotype induced indicate similar Ag stimulation of CD8 T cells in both groups.

The next important study was to determine if I.H. CD8 T cells could induce antiviral activity in response to antigenic re-encounter. We assessed the induced T cells’ ability to the produced antiviral cytokines, IFN-γ, TNF-α and IL-2, in response to ex vivo LCMV-GP33 peptide stimulation (Figure 2c). About 50, 55 and 13% of CD8 T cells from the lymph nodes of the I.H. group produced IFN-γ, TNF-α and IL-2, respectively.
TNF-α or IL-2, respectively, in this assay. These numbers were higher for SPL, where 77, 67 and 30% of CD8 T cells produced the above-mentioned cytokines. The LIV showed lower cytokine production at 44, 59 and 7.8%, respectively. In contrast, a higher percent of lymphoid-primed CD8 T cells produced these cytokines. For example, 93, 93 and 45% of CD8 T cells from the SPL of the I.M. control group produced IFN-γ, TNF-α or IL-2, respectively. Nevertheless, similarities in polyfunctionality (the ability of single cells to produce multiple cytokines) were observed in the SPL and LIV of the two groups. Although, cells from the lymph nodes of the I.H. group were significantly less functional than those of the I.M. group (Figure 2d).

In addition to antiviral cytokine production, a crucial function of effector CD8 T cells is induction of cytotoxicity. We stained the Ag-specific CD8 T cells for degranulation markers, granzyme B and CD107a, in order to verify whether these cells were functional effector CTLs. Both groups induced degranulation markers following Ag stimulation (Figure 2e). Accordingly, we used an in vivo cytotoxicity assay to assess the whether I.H.-immunized mice’s CD8 T cells became effective CTLs. One week after immunization, we examined the killing of transferred target cells pulsed with either LCMV-GP33 peptide or LCMV-NP (control). Twenty-four hours after transfer of target cells, about 88% of LCMV-GP33-pulsed target cells were eliminated in the blood of the I.H. group, but only 76% of the target cells were killed in the I.M. group (Figure 2f).

Intrahepatic immunization promptly establishes memory precursors effector CD8 T cells and maintains their memory phenotype. The ultimate goal of immunization is to generate strong effector cells to clear infection along with long-lasting memory CD8 T cells, which will quickly respond to subsequent infections. Recent studies have provided a phenotypic profile for a subset of effector cells.
Figure 2. Effector DbGP33⁺-specific CD8 T cells profile following intrahepatic (I.H.) immunization of P14 chemaric mice with LCMV-gp mutant. CFSE-labeled CD8 T cells isolated from lymphoid and non-lymphoid organs of mice either expressing Ag either I.H. or I.M. were analyzed at indicated time points. (a) Proliferative capacity and (b) expression of important activation markers on transferred DᵇGP33⁺-specific CD8 T cells. (c) The ability of I.H.-immunized CD8 T cells to produce cytokines: IFN-γ, TNF-α and IL-2, following ex vivo stimulation with synthetic peptide. (d) A representative flow chart showing IFN-γ-positive cells and their ability to coproduce TNF-α and IL-2 (pie chart). (e) Splenocytes from both groups (I.H. and I.M.) expressed degranulation markers (CD107a and GzmB). (f) GP33-specific CD8 T cells from both groups display rapid cytolytic activity during the first week of immunization. Data are representative of three independent experiments with four mice per group.
Figure 2. (Continued).
Figure 3. Effector and memory differentiation of activated CD8 T cells. Expansion and contraction of short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) in (a) PBMC and (b) other lymphoid and non-lymphoid tissues. (c) Distribution of effector and memory CD8 T cells from intrahepatically (IH)- and intramuscularly (IM) immunized mice at days 21 and 84 post infection. Data are representative of three independent experiments with four mice per group.
CD8 T cells that will commit to become memory T cells. Cells within a polyclonal response can be grouped into SLECs and MPECs based on their expression levels of the killer cell marker; KLRG1 among other surface markers. We investigated the difference in I.H. vs I.M.-primed CD8 T cells’ capacity to differentiate into SLEC and MPEC based on immunization route.

Figure 4. Effector CD8 T cell frequency magnitude and distribution. Frequency kinetics of the GP33-specific CD8 T-cell response in (a) PBMC and (b, c) lymph nodes, spleen (SPL), liver (LIV) and lung (LUN) following immunization. Data are representative of three independent experiments with four mice per group.
Activated CD8 T cells in I.H.-immunized mice experienced a swift expansion and contraction phase. Although CD8 T cells from both immunized groups differentiated into effector cells and memory cells, it was unclear whether they undergo this transition with the same frequency distribution. We explored the frequency of CD8 T-cell responses induced by I.H. in contrast to those induced by I.M. Following immunization, T-cell expansion from I.H. rapidly peaked on day 6 with 0.7% of Ag-specific cells detected in the blood. This early expansion was followed by a rapid contraction phase. At day 9 post infection, 57% of the Ag-specific cells were already dead and the percent contraction increased to 82% by week 2. In the I.M. group, only 0.2% Ag-specific cells were detected on day 6. However, an increase in Ag-specific CD8 T cells (to ~4.2%) was observed at day 13 post infection. Furthermore, the Ag-specific CD8 T cells in I.M. group underwent a gradual contraction over the next 3 weeks (Figure 4a).

To evaluate the tissue distribution of Ag-specific cells in lymphoid and non-lymphoid tissues, we calculated the absolute numbers of Thy1.1 cells in these tissues (Figures 4a and c). We observed similar expansion–contraction phases between groups in these tissues as seen in the blood (Figure 4a). The rapid and robust contraction phase in the I.H. group after expansion led to a low frequency of effector CD8 T cells, which persisted several weeks after immunization. The I.M. group on the other hand, exhibited ~1.6% of the activated cells still in the LIV, 12 weeks after immunization. The decrease in CTL frequency in I.H.-immunized mice led to defective viral clearance.

In order to examine the effects of these two different immune phenotypes in protection, we challenged the animals 3 weeks post immunization. At this time point 0.15% Ag-specific CD8 T cells can be detected in the SPL of the I.H.-immunized group (compared with 1.5% of the I.M. group). When killing assay was performed, <2% of the relevant Ag-pulsed target cells were eliminated in the lymph nodes. This is in sharp contrast to the I.M. group, which showed killing of about 75% of the target cells (Figure 5a). We further allowed the transferred target cells to reside in the recipient mice for up to 72 h, but we did not observe any improvement in killing (Figure 5a). This lack of cytolytic activity was also observed in the lymph nodes, SPL, LIV and LUN, illustrating that the vaccine-induced effector CD8 T cells were not selectively sequestered in any particular tissue (Figure 5b).

To confirm that the lack of target cell elimination in the I.H. group is not due to defective LIV-primed memory CD8 T cells, we transferred 3.5 × 10⁶ Ag-specific cells from each group into naive mice and evaluated their cytolytic activity in the recipient mice. Interestingly, none of the recipient groups were able to eliminate the peptide-pulsed target cells (Figure 5c).

DISCUSSION

The recent resurgence in popularity of synthetic DNA vaccination has been attributed to their ability to elicit strong humoral and cellular responses without the safety, stability and expense concerns associated with live attenuated and recombinant protein vaccines. It is therefore important to study this platform in the development of immune therapies for chronic infections such as HBV and HCV. These two hepatotropic pathogens are known to be the leading cause of one of the deadliest forms of cancer, HCC. Most of the experimental DNA vaccines have been delivered I.M. and they do not elicit significant immune responses in mucosal and/or other tissues that are not associated with the peripheral lymphoid tissues. It may be important to utilize the microenvironment of regional lymph nodes to present effector cells with homing instructions by using a delivery system that will make these regional lymph nodes the site of activation for immune cells. In this study, we report an unexpected defect in the initial induction of adaptive immunity following I.H. immunization of P14 chemagic mice. We believe that the unconventional priming of naive CD8 T cells in this type of immunization compromises the cells’ expansion capacity, resulting in a 3–500-fold decrease of effector cell frequency and 2–9-fold decrease of memory CD8 T-cell frequency in multiple tissues. Although T cells...
from this immunization group resulted in early effector differentiation, the overall quality of the effector functions, polyfunctionality and cytotoxicity, were somewhat comparable to CD8 T cells primed in lymphoid tissues. The memory cells' poor Ag clearance and poor viral control illustrate a low-CTL frequency.

Primary CD8 T-cell activation by liver cells have been described by several independent groups using in vivo and in vitro models.26,29,30 The fate of CD8 T cells primed in the liver has been a subject of much investigation in an effort to explain the failure of LIV protective immunity in chronic liver infections, such...
as HBV, HCV and malaria. Using liver transplantation and transgenic Ag models, Bertolino et al. suggested that liver-primed CD8 T cells underwent early Bim-dependent apoptotic death. Other groups studying this question with viral vectors, such as adenovirus and AAV, reported full differentiation with some defective antiviral activities. The outcomes of these studies are important in interpreting and understanding the present data. To gain insight into how Ag expression in the liver can help migrate effector T cells preferentially into the liver surroundings, and to compare the differentiation and memory programming of these T cells to normal I.M. immunization, a unique model that establishes Ag infection but eliminates chronic Ag expression in the liver, we used a hydrodynamic DNA plasmid injection approach to establish an acute liver infection model that allowed us to address this question. Hydrodynamic-based transfection normally results in the rapid uptake of DNA molecules into the cytosol of hepatocytes and detectable Ag expression in the liver within a few hours. The Ag expression kinetics are comparable to what is observed in skeletal muscle after conventional I.M. injection. We observed that such expression was restricted to the liver and lasted for ~4 days, mimicking acute infection. The level of transgene expression is likely to represent the expression of viral Ag in the liver during hepatotropic pathogenic infection. Furthermore, there was no significant elevation in blood alanine aminotransferase, an enzyme used to measure LIV damage.

Using this model, LCMV D\(^{\text{GP33}}\)–specific CD8 T-cell priming in P14 chimeric mice was confirmed by analysis of the upregulation of effector molecules. However, to clear pathogenic infection, CD8 T-cell priming alone is not sufficient. The activated CD8 T cell should be able to survive long enough to differentiate into a mature effector cell that possesses antiviral properties. After observing priming of CD8 T cells in both immunization groups, we observed rapid upregulation of pro-apoptotic markers (Supplementary Figure 1) in the I.H. group, and therefore believed that the CD8 T cells from the I.H. group may lack the ability to survive long enough to have a significant impact on viral resolution. This idea is based on the knowledge that Bim-dependent deletion of CD8 T cells is a link to the inability of HBV-specific CD8 T cells to control viral persistence in chronic infections. In the present study, we observed an early proliferative response of naïve CD8 T cells in the I.H. group when compared with the traditional I.M. immunization model, where T-cell activation is occurring in the draining lymph nodes. We noticed that the early cell division of Ag-specific CD8 T cells in the I.H. group was consistent with prior studies, which shows the kinetics of initial I.H. priming to be more rapid than by secondary lymphoid tissue-dependent priming. The early proliferation of D\(^{\text{GP33}}\)-specific CD8 T cells in the I.H. group was followed by significant untimely effector cell contraction. In this case, the CD8 T cells expanded early, but the cell frequency at their peak of expansion was several folds lower than CD8 T cells primed in the draining lymph nodes. As expected, the pro-apoptotic marker, Bim, was upregulated in the I.H. group during this contraction phase. The Bim levels in the I.M. group eventually caught up with the I.H. group during its peak of expansion (Supplementary Figure 1A). Interestingly, the survival signal, Bcl-2, in the I.H. group was downregulated in the first week when Bim was high, but increased during the second and third weeks post immunization (Supplementary Figure 1B). Although this data support that CD8 T cells from I.H. immunization, albeit hastily, experience the same death and survival phases as CD8 T cells from I.M. immunization, it also provides an inkling of induction of a different CD8 T-cell activation pattern.

In addition to the phenotypic examination I.H. and I.M. CD8 T cells’ effector differentiation status, we also analyzed their functional properties. Although some prior studies reported full functional differentiation of CD8 T cells when Ag is expressed in the liver, a more comparative analysis of these effector cells disclosed defective antiviral activity. Our findings show that the effector CD8 T cells from the I.H. group can secrete antiviral cytokines. Though they were inferior single producers, their polyfunctionality, a property reported to correlate with protection in specific models, was very similar to the control group in all tissues except the lymph nodes. It is noteworthy that for both immunized groups effector CD8 T cells recovered from the LIV were less functional compared with those found in the same animals’ spleen and lymph nodes. Therefore, prior work and the data presented here support that the liver environment has a crucial role in effector T-cell suppression. At earlier time points, the I.H. group mounted a strong cell-mediated cytotoxic effect upon Ag re-encounter. This data support that effector CD8 T cells recovered from I.H. immunization early can be as effective as those from traditional I.M. immunization.

Our results also reveal that a subset of effector cells (MPEC), which are likely to become long-lived memory cells, can be detected in the I.H.-immunized group’s effector CD8 T-cell population as early as 1 week post immunization. Although CD8 T cells from I.H. differentiate into memory cells quicker than I.M.-immunized group, the distribution of effector, effector-memory and central memory cells were similar in all tissues within the groups.

We evaluated recall and effector differentiation of these long-lived memory CD8 T cells in both lymphoid and non-lymphoid tissues. The I.M. group responded robustly and eliminated target cells from all tissues upon secondary challenge. In contrast, the I.H. group was deficient in killing these Ag-pulsed target cells. This lack of killing was likely associated with the low frequency of effector-memory cells in the I.H. group. Although the I.H. group’s
memory precursors comprise about 16% of peak effector cells in the I.H. group, the frequency was still very low because of its lower effector frequency. In support, hydrodynamic-immunized mice repeatedly succumbed to lethal dose LCMV I.C. challenge.

To investigate the difference we observed in the expansion–contraction phase and the decrease in frequency in the I.H. group, we examined differences in the initial induction of CD8 T cells in both groups. Using models that ensure the exclusion of CD8 T-cell priming in lymphoid tissues, we observed proliferation and expansion of GP33+/Thy1.1 cells in P14 chimeras after I.H. immunizing mice with cognate viral Ag. Collectively, these data show that hydrodynamically injecting DNA plasmid into mice causes lymphoid-independent CD8 T-cell activation.

In summary, these results provide evidence that the lack of recall response needed to protect mice from secondary challenge after I.H. immunization is directly associated with a defect in the initial induction of the Ag-driven response. This correlation can be made to human studies in which the lack of viral clearance in chronic HBV and HCV infections have been linked with the low number of HBV or HCV-specific CD8 T cells detected in the blood. Although further studies are needed, the data support that it is important to concentrate on improving traditional I.M. immunization that will preferentially traffic effective cellular immune cells into the liver for immunotherapies to target hepatotropic pathogens.

ABBREVIATIONS
HBV, hepatitis B; HCV, hepatitis C; HI, hydrodynamic injection; I.C., intracranial; I.H., intrahepatic; I.M., intramuscular; LIV, liver; MPEC, memory precursor effector cells; SLEC, short-lived effector cells;

CONFLICT OF INTEREST
DBW has grant funding, participates in industry collaborations, has received speaking honoraria and fees for consulting. This service includes serving on scientific review committees and advisory boards. Remuneration includes direct payments or stock or stock options and in the interest of disclosure; therefore, he notes potential conflicts associated with this work with Pfizer, Bristol Myers Squibb, Inovio, Merck, VGXI, Aldevron and possibly others. Licensing of technology from his laboratory has created over 100 jobs in the private sector in the biotech/pharma industry. The other authors declare no competing financial interests.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)