MINI REVIEW

Hepatic effector CD8⁺ T-cell dynamics

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CD8⁺ T cells play a critical role in hepatitis B virus (HBV) pathogenesis. During acute, self-limited infections, these cells are instrumental to viral clearance; in chronic settings, they sustain repetitive cycles of hepatocellular necrosis that promote hepatocellular carcinoma development. Both CD8⁺ T-cell defensive and destructive functions are mediated by antigen-experienced effector cells and depend on the ability of these cells to migrate to the liver, recognize hepatocellular antigens and perform effector functions. Understanding the signals that modulate the spatiotemporal dynamics of CD8⁺ T cells in the liver, particularly in the context of antigen recognition, is therefore critical to gaining insight into the pathogenesis of acute and chronic HBV infection. Here, we highlight recent data on how effector CD8⁺ T cells traffic within the liver, and we discuss the potential for novel imaging techniques to shed light on this important aspect of HBV pathogenesis.

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Our understanding of hepatitis B virus (HBV) immunopathogenesis has greatly improved in recent years.^{1–3} It is clear that HBV replicates non-cytopathically in the hepatocyte and that both viral clearance and most of the clinical syndromes associated with this infection reflect the adaptive immune response, particularly the virus-specific CD8⁺ T-cell response.^{1–3} Despite these accomplishments, however, a number of fundamental issues pertaining to the pathogenesis of this disease remain unanswered, including the means by which HBV-specific effector CD8⁺ T cells traffic and recognize antigen within the liver and how such processes are affected by the anatomic and hemodynamic changes that characterize the complications of chronic HBV infection (i.e., fibrosis/cirrhosis and hepatocellular carcinoma).

The classic paradigm for leukocyte migration from blood vessels to interstitial tissues involves a multistep process that occurs in post-capillary venules,⁴ but not in arterioles or capillaries (where leukocyte adhesion may limit gas exchange and tissue perfusion⁵). The initial weak rolling interactions between leukocytes and endothelial cells are mediated by a family of proteins called selectins.⁶ There are three types of selectins: one expressed on leukocytes (L-selectin), one expressed on endothelial cells (E-selectin) and one expressed on both platelets and endothelial cells (P-selectin). The ligands for selectins are sialylated oligosaccharides bound to mucin-like glycoprotein

backbones.⁶ Firm adhesion of leukocytes to endothelial cells is mediated by a family of heterodimeric leukocyte surface proteins called integrins.^{4,7} The combination of cytokine-induced endothelial expression of integrin ligands, mainly vascular cell adhesion molecule 1 and intercellular adhesion molecule 1, and chemokine-mediated conversion of integrins to a high-affinity state on leukocytes⁸ results in firm adhesion of leukocytes to the endothelium at sites of inflammation.

The liver represents an exception to this leukocyte migration paradigm in several respects.^{9,10} First, leukocyte adhesion is not restricted to the endothelium of post-capillary venules, but it also occurs in sinusoids.¹¹ Remarkably, the quantitative importance of sinusoidal adhesion is less established for CD8⁺ T cells, particularly in the context of intrahepatic antigen recognition. Second, while in post-sinusoidal vessels, rolling precedes adhesion, and leukocyte adhesion to liver sinusoidal endothelial cells (LSECs) often occurs independent of any notable rolling.⁹ Notably, LSECs lack both tight junctions between cells and a basal membrane.¹² This is in contrast to most vascular beds in other tissues and organs, where a continuous endothelial cell layer and a basement membrane physically separate parenchymal cells from circulating leukocytes.¹² Moreover, hepatocyte and white blood cell villi can protrude through the fenestrated endothelial barrier of sinusoids, thus providing the opportunity for direct interaction of circulating

cells with the underlying hepatocytes.¹³ For all of these reasons, the molecular mechanisms leading to leukocyte adhesion to LSECs appear to differ from those occurring in post-capillary venules of other vascular districts.^{9,10}

The mechanisms by which effector CD8⁺ T cells traffic within the liver are incompletely understood and have been under investigation in our laboratory over the last few years. Several recent studies have demonstrated that the intrahepatic recruitment of antigen-specific effector CD8⁺ T cells is critically dependent on platelets.^{2,14–18} Indeed, using mouse models of CD8⁺ T cell-mediated acute viral hepatitis, we have recently shown that platelet depletion is associated with a profound reduction in the intrahepatic accumulation of virus-specific effector CD8⁺ T cells and a proportional reduction in liver disease severity.¹⁴ Both phenotypes are restored upon reconstitution with normal platelets, but not upon reconstitution with platelets treated with prostaglandin E₁, a known inhibitor of platelet activation.¹⁴ *In vitro* findings also indicate that under the low shear flow conditions likely occurring in the venous circulation of the liver, antigen-specific effector CD8⁺ T cells tightly interact with platelets, and similarly, this process is inhibited when platelets are treated with prostaglandin E₁.¹⁴ In an ongoing effort to explain mechanistically why platelets are required to support CD8⁺ T cell-induced liver pathology, we also found that this process is influenced by two specific inhibitors of platelet activation pathways, aspirin (that blocks thromboxane A₂ production) and clopidogrel (that blocks the P2Y₁₂ ADP receptor¹⁹). Indeed, treating mice with aspirin, clopidogrel or a combination of the two, attenuates acute and chronic liver injury by reducing the hepatic accumulation of antigen-specific CD8⁺ T cells and antigen-nonspecific inflammatory cells.^{15,18} Whether a functional connection between platelets, T cells and sinusoidal endothelial cells depends on direct and/or indirect intercellular interactions within the liver remains to be demonstrated. One interesting molecular candidate for such an interaction is CD44, which has been shown to control neutrophil accumulation within liver sinusoids²⁰ and is expressed by both effector CD8⁺ T cells and platelets (manuscript in preparation).

Although the liver is amenable to intravital microscopy,^{21–24} the visualization of CD8⁺ T cell-induced liver immunopathology in animal models has been limited to static imaging of sectioned tissues. This approach is inadequate to unravel potential hemodynamic and anatomic constraints regulating CD8⁺ T-cell function in the liver. Moreover, systematic *in vivo* measurement of intrahepatic blood flow velocity at the level of individual sinusoids has, to our knowledge, never been performed. Indeed, current techniques for measuring blood flow *in vivo* lack the necessary spatial resolution or require high concentrations of large probes that might alter blood flow (see below). Recent technological advances in wide field epifluorescence and multiphoton intravital microscopy, along with specific features of fluorescence correlation spectroscopy and correlative confocal and transmission electron microscopy, provide, at last, the opportunity to visualize liver immunopathology as it progresses *in vivo*.

Epifluorescence intravital microscopy²⁵ allows for the study of rapid intravascular events at video-rate (greater than 10 frames/second at full resolution in our current setup) and permits the detection of relatively faint fluorescent signals. However, it has limited resolving power and it cannot provide accurate spatial information in three dimensions. Although the ability of epifluorescence intravital microscopy to image fluorescent events that occur below the surface of a solid organ is inevitably restricted by scattering and absorption of excitation and emission light, it is not particularly relevant for the liver, as the surface of this organ is representative of the whole. Thus, whereas epifluorescence intravital microscopy is a powerful tool to analyze intravascular leukocyte behavior, such as effector CD8⁺ T-cell adhesion to LSEC (Ref. 26 and Supplementary Movie 1) or platelet–T cell interactions, its usefulness for observations of extravascular events such as transendothelial or interstitial migration or cell–cell interactions is more limited.

These shortcomings are overcome by multiphoton intravital microscopy.²⁵ This technique has several advantages over conventional epifluorescence microscopy: first, infrared excitation light penetrates deeper into organic matter, thus allowing non-invasive observations far below the surface of thick samples; second, because multiphoton effects occur only in the objective's focal point, there is no bleaching of fluorescent dyes above or below that point allowing for long-term imaging of biological processes; third, much less light energy is absorbed by tissues, leading to greatly reduced phototoxicity; and finally, all systems are equipped with highly sensitive external non-descanned detectors, which can generate images from very faint light signals in up to four different colors simultaneously in our current setup. Thus, multiphoton intravital microscopy is best suited to study hepatic T cell behavior after their adhesion to endothelial cells (Supplementary Movie 2), including extravasation and eventual interstitial migration.

Correlative confocal and transmission electron microscopy²⁷ combines the high resolution of electron microscopy with the specificity of fluorescence labeling. Although it does not have live imaging capability, we have adapted this technique to the liver tissue (Figure 1), and we are currently assessing whether effector CD8⁺ T cells can recognize hepatocellular antigen and perform effector function in a diapedesis-independent manner.

As mentioned earlier, current techniques for measuring blood flow velocity *in vivo* lack the necessary spatial resolution to provide information at the level of individual sinusoids (laser speckle imaging,^{28,29} optical Doppler tomography^{30,31} and magnetic resonance imaging) or adopt analysis protocols that are restricted to simple geometries (particle image velocimetry^{32,33}). To overcome these limitations, several optical methods have been developed to quantify the blood flow velocity within microvessels by exploiting the concepts of fluorescence correlation spectroscopy and fluorescence cross-correlation spectroscopy, well-established techniques based on the analysis of fluorescence intensity fluctuations due to molecules or cells diffusing or flowing through tiny observation

Figure 1 Correlative confocal and transmission electron microscopy. Electron micrograph (top), fluorescence micrograph (middle) and overlay image (bottom) of the liver of a mouse that was transferred with HBV-specific effector CD8⁺ T cells (red). Green denotes LSECs. Scale bars=2 μm. HBV, hepatitis B virus; LSEC, liver sinusoidal endothelial cell.

volumes.^{34–37} Fluorescence correlation spectroscopy and fluorescence cross-correlation spectroscopy offer several advantages such as high spatial resolution (approximately 1–2 μm), the ability to measure a wide dynamic range of flow velocities³⁸ and high sensitivity (free fluorescent dyes can be readily used as tracers at pico- to nano-molar concentration ensuring minimal to no flow disruption).^{39,40} Recently, an electron multiplying charge-coupled device dual-beam cross-correlation setup has been exploited for the measurement of blood flow velocity *in*

vivo by computing the cross-correlation function of fluorescent red blood cells.⁴¹ A single line-scanning cross-correlation method (coupled with two-photon excitation) has also been implemented, in which a laser beam is scanned along the blood vessel central axis at a high frequency (approximately 1–10 kHz), allowing the quantification of blood flow velocity at the level of a single capillary⁴² and the measurement of the strain rate.⁴¹

In collaboration with the Department of Physics at the University of Milano-Bicocca (Milan, Italy), we are developing a novel flow measurement technique relying on a column-based correlative analysis of single raster scanned images. This method, applicable to both confocal and two-photon *in vivo* microscopy, allows mapping of blood flow velocity at a high spatial and time resolution in the complex network of vessels constituting the hepatic microcirculation while simultaneously maintaining the morphologic information related to the dynamic parameters of vessels and immune cells (manuscript in preparation). We believe that this method will allow us to assess important unanswered questions such as the extent to which altered hepatic blood flow impacts CD8⁺ T-cell vascular adhesion (and the extent to which CD8⁺ T-cell vascular adhesion alters normal hepatic blood flow).

In conclusion, while some of the rules that govern CD8⁺ T-cell homing to the liver have begun to be clarified at the population level, we still have limited knowledge of the precise dynamics of hepatic CD8⁺ T-cell migration and its interaction with other cell types at the single-cell level, particularly in the context of intrahepatic antigen recognition. We believe that recent advances in the field of live imaging, coupled with animal models that express viral antigens in the hepatocyte, will provide an opportunity to tackle some of these questions directly in a living animal. This will not only improve our understanding of CD8⁺ T-cell trafficking within the liver, but may also provide tools for the design of new therapeutic strategies for the treatment of chronic viral hepatitis and liver cancer.

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