Heterogeneity of HBV-Infected Hepatocytes in Humanized Chimeric Mice Revealed by Single Cell Analyses Using FACS and Duplex Digital PCR

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Introduction and Methods

HBV-infected hepatocytes produce vast amounts of HBsAg particles, typically exceeding virion output by ≥1,000:1. These ratios likely vary among individual cells depending on their efficiency in transcribing S versus pregenomic RNAs. We hypothesize that HBV-infected livers contain heterogeneous populations with varying levels of viral components, which may explain clinically observed patterns of antiviral response.

Liver cells were isolated from uninfected and HBV-infected chimeric mice with humanized livers, stained with FITC-conjugated anti-HBs antibody, and analyzed by FACS. HBsAg-positive cells with different side scatter (SSC) values were single-cell sorted into 96-well plates and analyzed using Absolute Q™ Duplex Digital PCR to simultaneously detect cccDNA and rcDNA.

Results

1. Increased size and granularity were detected in a portion of HBV infected cells isolated from humanized livers of chimeric mice

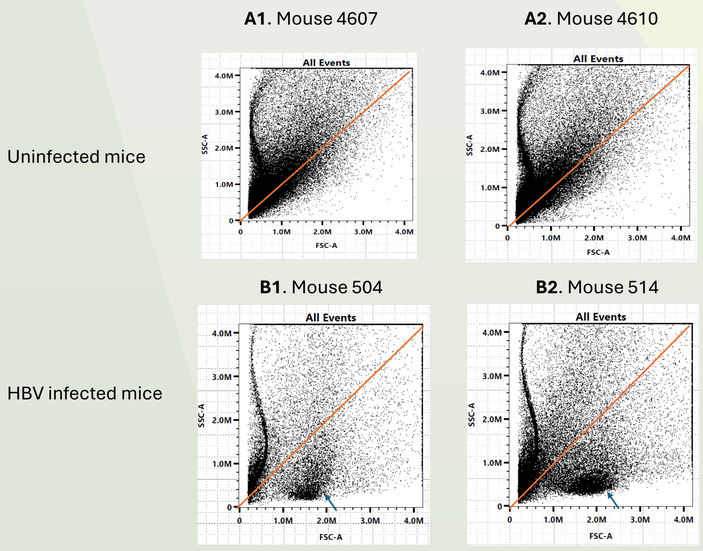

Figure 1. FACS analysis shows wider distribution pattern of HBV infected cell population compared to uninfected cells. Human liver cells were isolated from 4 humanized livers of chimeric mice through perfusion and subjected to FACS analysis. Cell populations are plotted on side scatter (SSC) and forward scatter (FSC). **A**. Distribution pattern of uninfected cells from two mice. **B**. Distribution pattern of infected cells from two mice. A red diagonal line is added to show a portion of infected cells was distributed in right diagonal region. A blue arrow points to a dense infected cell population, which was absent among uninfected cells.

2. Strategy to gate and sort infected cells with different SSC values for duplex dPCR analysis

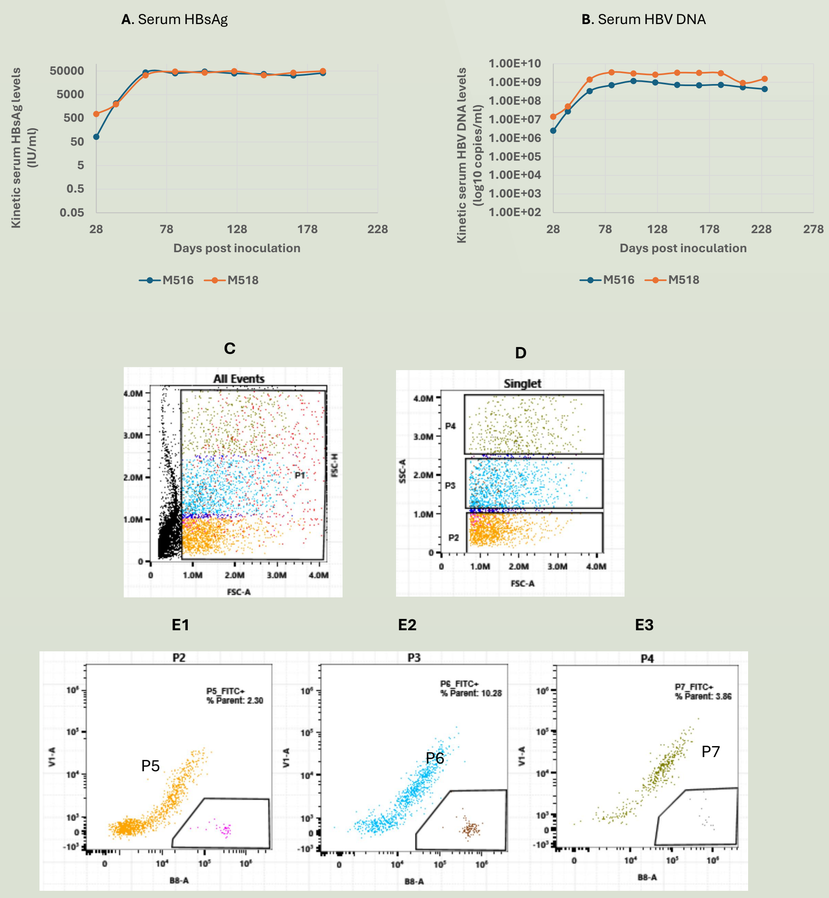

Figure 2. Sort HBsAg positive cells isolated from two chimeric mice on day 228 pi. **A** and **B**. Kinetic levels of serum HBsAg and HBV DNA in mice 516 and 518. **C**. Distribution pattern of human liver cells isolated from infected mouse 518. **D**. Singlets from mouse 518 were further gated into P2-P4 based different SSC values. **F**. HBsAg positive cells, i.e. FITC positive cells from P2-P4 were further gated into P5-P7, respectively. Cells from P5 and P6 were individually sorted and deposited into wells of 96-well plates for duplex dPCR analysis

3. Cells with higher SSC values are associated with cells with higher FITC or HBsAg intensities (Table 1)

Mouse ID	Mean FITC intensity of HBsAg positive cells in 3 gated populations		
	P5	P6	P7
516	5.39E5	1.27E6	2.61E6
518	4.6E5	9.99E5	2.66E6

Conclusion

HBV-infected human hepatocytes display marked heterogeneity in size and granularity by FACS analysis, suggesting varying levels of viral components among individual cells. Further characterization of these distinct infected populations may help elucidate the relationships between RNA transcription patterns, cccDNA status, and their responses to antiviral therapy.

Discussion

These results reveal substantial heterogeneity among HBV-infected hepatocytes, with distinct intracellular viral DNA profiles and variable levels of HBsAg. This may contribute to clinical patterns of >4-log HBV DNA reductions with minimal HBsAg decline during ALT flares (10.1056/NEJMoa2210027), where damaged cells likely harbor high levels of virions but low HBsAg, resembling the HBsAg-/HBc+ cells. This cell population also offers an alternative explanation to the cccDNA longevity model for the observed disparity between ≥5-log HBV DNA and <1-log HBsAg reductions after 8 years of TAF/TDF therapy (10.1111/apt.18278). Additionally, HBsAg+/cccDNA+/rcDNA− cells identified here suggest that some HBsAg+/HBc− cells may still retain cccDNA.

4. Two types of cells: cccDNA-/rcDNA+ and cccDNA+/rcDNA- cells were detected among HBsAg positive cells

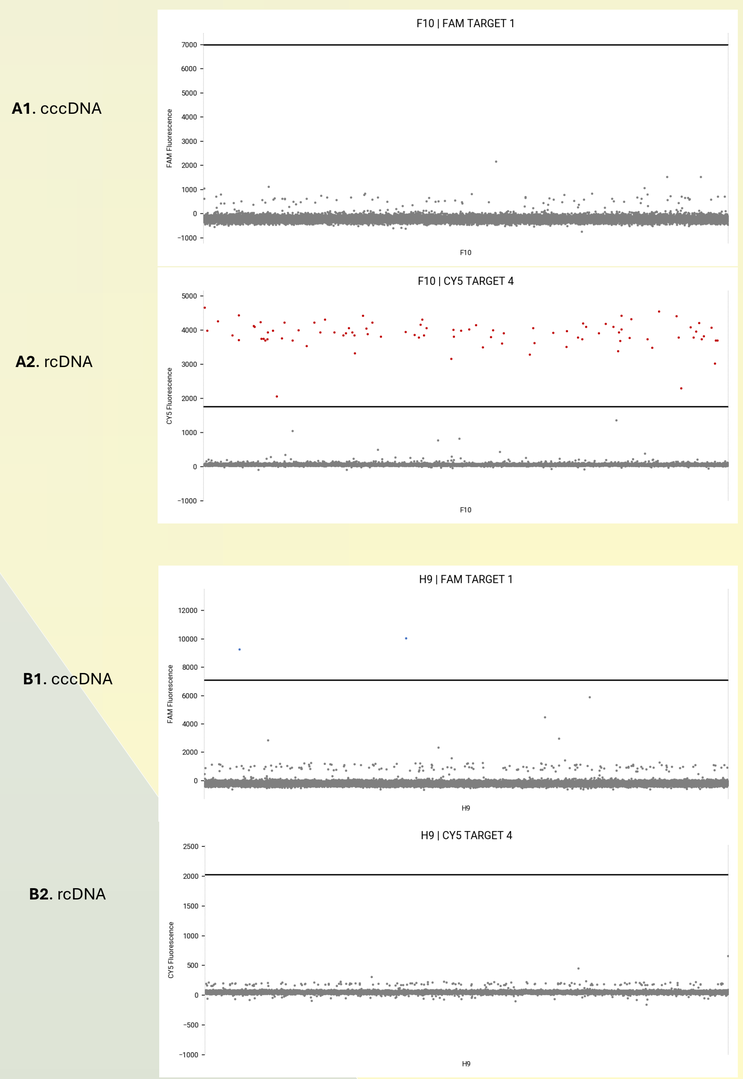

Figure 3. Absolute Q duplex digital PCR (ABQ duplex dPCR) detection of both cccDNA (blue) and rcDNA (red) in the same HBsAg positive cells. HBsAg positive cells were singly sorted and deposited into wells of 96-well plates. Released DNA from the deposited cells is mixed with dPCR master mix and two probes, i.e. cccDNA probe labeled with FAM and rcDNA probe labeled with CY5. The data readout is generated by ABQ dPCR software v2.4.16. **A**. An example of HBsAg +/cccDNA -/rcDNA+ (94 copies) cell. **B**. An example of HBsAg +/cccDNA + (2 copies)/rcDNA - cell.

5. The cccDNA status in HBsAg positive cells differed significantly between the P5 and P6 populations (Table 2)

Table 2. Duplexing detection of cccDNA and rcDNA in single HBsAg positive cells

	Cells with cccDNA only detected	Cells with rcDNA only detected	Cells with both cccDNA and rcDNA	Total
P5 (lower SSC)	28 (25%)*	74 (67%)*	8 (7%)	110
P6 (higher SSC)	68 (78%)*	13 (15%)*	7 (7%)	87

* Chi-square value (X^2) is 55.54 and p-value is 9.14×10^{-14} in comparison of the differences in the detected cccDNA only and rcDNA only containing cells between P5 and P6 populations.

Acknowledgement

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