Ex vivo Analysis of Resident Hepatic Pro-inflammatory CD1d-reactive T cells & Hepatocyte Surface CD1d Expression in Hepatitis C

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Abstract

Hepatic CD1d-restricted and natural killer T cell populations are heterogeneous. Classical ‘Type 1’ α-galactosylceramide-reactive CD1d-restricted T cells express ‘invariant’ TCRα (‘iNKT’). iNKT dominating rodent liver are implicated in inflammation, including in hepatitis models. Low levels of iNKT are detected in human liver, decreased in subjects with chronic hepatitis C (CHC). However, high levels of human hepatic CD161⁺CD56⁺ non-invariant pro-inflammatory CD1d-restricted ‘Type 2’ T cells have been identified in vitro. Unlike rodents, healthy human hepatocytes only express trace and intracellular CD1d. Total hepatic CD1d appears to be increased in CHC and primary biliary cirrhosis.

Direct ex vivo analysis of human intra-hepatic lymphocytes (IHL), including matched ex vivo versus in vitro expanded IHL, demonstrated detectable non-invariant CD1d-reactivity in substantial proportions of HCV-positive livers and significant fractions of HCV-negative livers. However, α-galactosylceramide-reactive iNKT were detected only relatively rarely. Liver CD1d-restricted IHL produced IFNγ, variable levels of IL-10, and modest levels of Th2 cytokines IL-4 and IL-13 ex vivo. In a novel FACS assay, a major fraction (10–20%) of hepatic T cells rapidly produced IFNγ and up-regulated activation marker CD69 in response to CD1d. As previously only shown with murine iNKT, non-invariant human CD1d-specific responses were augmented by IL-12. Interestingly, CD1d was also found selectively expressed on the surface of hepatocytes in CHC, but not those CHC subjects with history of alcohol usage or resolved CHC. In contrast to hepatic iNKT, non-invariant IFNγ-producing Type 2 CD1d-reactive NKT cells are commonly detected in CHC, together with cognate ligand CD1d, implicating them in CHC liver damage.

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Disclosure
Authors declare no competing interests.
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Introduction

Chronic hepatitis C (CHC) virus (HCV)-infection involves immune-mediated liver destruction, although immunity also controls viral replication (1–8). HCV profoundly influences immunity (1–10). ~30% of human intra-hepatic lymphocytes (IHL) co-expressing T and natural killer (NK) cell proteins are frequently activated/memory and MHC-restricted (1–5,8,9;11–16). However, a major fraction of in vitro-cultured IHL is CD1d-restricted (5,8,9;16–22). Increased hepatic CD1d and CD1d-reactivity were reported in CHC (5,8,9;16–22). Increased Th2/pro-fibrotic CD1d-reactive cytokines are found in cirrhosis (20,21). However, most functional studies were in vitro, well-established for conventional T cells, but un-validated for CD1d-reactivity.

MHC-like non-polymorphic CD1d is constitutively expressed by myeloid and B cells as well as in the gut (23–25). Low level CD1d is apparently expressed inside healthy human hepatocytes (26), up-regulated in CHC and primary biliary cirrhosis (PBC) (21,22,27). CD1d is constitutively expressed on rodent hepatocytes (23,24,28). Possibly related to CD1d differences, high prevalence of rodent hepatic invariant TCR+ ‘NKT’ subset (iNKT; ‘Type 1’) compares to rare human liver iNKT, which are further reduced in CHC (5,8,9;16–22,29–32). While rare in human blood (~0.1%), iNKT are less frequent in matched liver (18). Also, human in vitro-cultured healthy hepatic CD1d-reactive NKT cells are Th1-biased (19,21,22), whereas rodent iNKT are Th1/Th2 (5,8,9;29–32). Hence, caution is needed in extrapolating the rodent CD1 system to humans.

Potent CD1d-restricted NKT cell ability to promote Th1 responses and/or CD1d-specific cytotoxicity contribute to resistance against certain infections and tumors through dendritic cell/macrophage maturation and IL-12, leading to activation of NK, B, and T cells (5,8,9,19;29–39). CD1d-reactive NKT rapidly secrete large amounts of Th1, Th2, Treg, and/or Th17 cytokines, depending on origin and health status (5,8,9;19–22,29–32).

iNKT cells express invariant Valpha, limited Vbetas, and NK receptors (29–33). iNKT specifically respond to CD1d-presented alpha-galactosylceramide (αGalCer) (29–32). CD1d responses are not always protective. Several bacterial genera make αGalCer-related iNKT-ligands, including Koch’s postulate-like demonstration of iNKT recognition of Novosphingobium lipid in PBC (27,34,35). Although functionally similar to iNKT, ‘non-invariant’ CD1d-restricted T cells (‘Type 2 NKT’) use diverse TCR. Indeed, recognition of up-regulated CD1d by murine Vγ4+ T cells causes viral myocarditis, an autoimmune sequela of otherwise successful picornaviral immunity (40,41). Murine iNKT can cause acute hepatitis (42–45). However, αGalCer suppresses viral replication and phenotypically NKT are activated in HBV models (46,47).

CD1d is expressed on human liver mononuclear cells and unlike other CD1s, CD1d-reactivity is high in uninvolved liver of wedge biopsies (22). Using surgical specimens, we
now report low level iNKT activity, but a high proportions of hepatic CD1d-reactivity demonstrated ex vivo from CHC subjects and from a proportion of controls. CD1d recognition by IHL from HCV+ donors produced prototype inflammatory IFNγ, variable IL-10, and detectable Th2 cytokines. Interestingly, hepatocyte surface CD1d was also markedly elevated, specifically in CHC. Results suggest that resident hepatic non-invariant CD1d-restricted NKT respond to increased hepatocyte CD1d in CHC, with potentially pathologic consequences.

Material & Methods

Study Subjects

Discarded liver tissue surplus to pathology were obtained from patients with ESLD/liver failure due to amyloidosis, autoimmune or viral hepatitis, primary sclerosing cholangitis, and/or alcohol abuse (Table 1). Cirrhotic transplant recipient ESLD/FHF subjects reflected this demographic (21–62 yo.; mostly US Veteran males, late 40s–mid-50s). Non-ESLD control liver samples were from similar subjects with primary HCC or metastatic (primarily documented or presumed colonic) tumors obtained from Cooperative Human Tissue Network or National Disease Resource Interchange. Studies were approved by the institutional Committee on Clinical Investigations.

Reagents

Antibodies, including CD1d-specific mAbs, and blood-derived iNKT controls, and human mock and CD1d transfectants were described (21,22,25,36, 24,28,33). mAb were from eBioScience, Inc., except the cytokine capture reagents from Miltenyi Bio., Inc. (Table 2).

Isolation of intra-Hepatic lymphocytes (IHL), FACS, & CD1d Functional Assays

To obtain IHL, surgical samples were minced to ~2mm, passed through 70µM sieve and subjected to standard Percoll gradient centrifugation. Where noted, small fractions of IHL were expanded in vitro, as previously (19,21), to directly compare to ex vivo. Media: RPMI-1640, 10% fetal bovine serum, antibiotics, 20UI/mL IL-2 (NIH AIDS Reagent Resource). Briefly, CD1d-reactive proportions were determined as previously (19,21,22,33,48,49) by incubating IHL or iNKT with CD1d+ or Mock C1R transfectants at 1:1 ratio (50,000/well) with phorbol myristic acid (PMA; 1ng.mL−1; ‘Total CD1d’=CD1d - Mock), or IL-12 (10ng.mL−1) (50). Cytokines were measured by ELISA (Endogen, Inc.), limit 1ng.mL−1. Standard error of means shown. Cytokine capture FACS was performed after 4hr. stimulation and with CD8, CD69 and IFNγ mAb (Table 2), gating on lymphocytes using FC500 (Beckman-Coulter), as described (19,21). FACS analysis was gated on lymphocytes (Fig. 3) or large hepatocytes (Fig. 4) from the same liver samples.

Results

Comparison of hepatic CD1d-reactive T cells assayed ex vivo versus after in vitro expansion

CD1d-reactivity (predominantly IFNγ) is detectable in the majority of human liver biopsy samples assayed after in vitro expansion, from wedge biopsy lymphocytes assayed from
healthy liver transplant donors, and from uninvolved tissue of tumor resections \textit{ex vivo} \cite{19,21,22}. To test the validity of these findings, IHL from a range of donors were directly tested \textit{ex vivo} compared to responses of similar liver samples after expansion \textit{in vitro} (Figure 1A,B). A range of modest to strong (>100pg.mL\(^{-1}\)) net CD1d-specific (CD1d\(^{+}\)–Mock C1R) IFN\(\gamma\) responses were detected from directly \textit{ex vivo}-assayed IHL (Figure 1A), which, when normalized, represented 5–10\% of quality control mitogen (PHA) responses for the majority of positive IHL (Figure 1B). CD1d responses of IHL \textit{ex vivo} were comparable to levels obtained with \textit{in vitro} expanded IHL, although as expected, mostly less than from anonymous leukopak-derived pure iNKT cell line controls \cite{19,21,22} assayed at the same cell numbers (Figure 1A–E).

Given these results, IHL were directly tested \textit{ex vivo} compared to responses obtained from matched liver samples after expansion \textit{in vitro}. Again, although responses were somewhat lower on a per cell basis than from matched \textit{in vitro} expanded IHL, direct \textit{ex vivo} assayed material contained clear CD1d reactivity (Figure 1C–E).

We further analyzed cytokines known to be produced by blood iNKT \cite{33} as well as some CD1d-restricted IHL lines \cite{19,21,22}. Most IHL produced little or no IL-4 to CD1d \textit{ex vivo}, although significant amounts \textit{in vitro}, as previously \cite{20,21} and to mitogen (limit of detection 1ng.mL\(^{-1}\)) (Figure 1D,E). Variable, but significant levels of CD1d-dependent IL-10 were produced (Figure 1A–C). Interestingly, unlike other cytokines, CD1d IL-10 levels, while variable, were comparable to mitogen (Figure 1E), suggesting a large proportion of human liver IL-10-producing cells were CD1d-reactive.

\textbf{Non-invariant-type hepatic CD1d-reactive T cells are frequently detectable from HCV-infected and negative subjects \textit{ex vivo}}

To determine the specificity of net CD1d responses observed \textit{ex vivo}, control or CD1d mAb was included in assays. As shown in Figure 2A, 2–10-fold of IHL and iNKT CD1d reactivity was specifically inhibited by CD1d mAb, similar to previous \textit{in vitro} results of IHL and other CD1d-reactive NKT \cite{19,21,22,33}.

We next determined whether the presence of Th1-like hepatic CD1d-reactive T cells assayed directly \textit{ex vivo} or as matched cell lines represented \(\alpha\)GalCer-specific iNKT. Only 3/28 IHL showed significant \(\alpha\)GalCer-specific iNKT IFN\(\gamma\) production, compared to 9/28 total CD1d-reactive and 1/10 \(\alpha\)GalCer-reactive HCV\(^{+}\) subjects, compared to 4/10 total CD1d-reactive (Figures 2B,C,E,F). As expected, control iNKT total IFN\(\gamma\) CD1d-reactivity was comparable to \(\alpha\)GalCer responses (Figure 2B,C). Since IHL IFN\(\gamma\) responses to \(\alpha\)GalCer were less frequent than total CD1d-reactivity, such reactivity was not mainly due to iNKT.

iNKT produce large amounts of IL-4 \cite{29–33}. \textit{Ex vivo} IHL IL-4 CD1d reactivity was relatively rarely detected, only 2/26 samples tested producing detectable CD1d-specific IL-4 (>1pg.mL\(^{-1}\)), although mitogen demonstrated potential of some liver T cells to produce IL-4 (Figures 1D,2D). This reflects overall Th1 bias of human hepatic T cells \cite{1–9,17}. IHL IL-4 total CD1d-reactivity appeared to be more closely \(\alpha\)GalCer-induced and iNKT-related, since where produced, these were of a similar fraction to each other (both ~10\% of mitogen;
Control iNKT cell lines derived from healthy subject blood produced \(>100\text{pg.mL}^{-1}\) IL-4 in response to CD1d, \(\alpha\text{GalCer}\), and to mitogen (Figure 2D).

To further evaluate levels of non-invariant-type hepatic CD1d-reactive T cells \textit{ex vivo}, we examined HCV\(^\pm\) IHL directly \textit{ex vivo} from healthy liver or with a range of diseases (transplant recipients, viral/non-viral fibrosis/cirrhosis, tumor-bearing, etc.; Table 1; Figure 2). Although there were relatively few inflammatory cells obtained from healthy controls and from cirrhotics, IHL were obtained using available larger samples. Such IHL frequently contained readily detectable CD1d-reactivity \textit{ex vivo}, whether HCV\(^+\) or HCV-negative (Table 1; Figures 1,2).

Overall, 32\% (9/28) of liver samples tested \textit{ex vivo} demonstrated CD1d-reactivity. 5/14 HBV/HCV-negative and 0/3 HBV\(^+\) subjects produced significant levels of CD1d-specific IFN\(\gamma\). 1/5 IHL from HCV\(^+\) subjects with documented history of alcohol abuse and 3/5 other HCV\(^+\) IHL produced readily detectable CD1d IFN\(\gamma\) responses (Figure 2E,F; Table 1). Measurable CD1d-reactivity of HCV\(^+\) IHL was 7, 20, and 59\% of mitogen IFN\(\gamma\) responses (Table 1), comparable to HCV-negative subjects (median=34\% of mitogen; range: undetectable-comparable to mitogen). Finally, significant IL-13 could be detected in response to CD1d from some subjects \textit{ex vivo} (Figure 2G), consistent with modest levels detected from \textit{in vitro} IHL cultures (19).

In summary, \textit{ex vivo} results were consistent with our previous results of a substantial population of largely non-invariant Th1-biased human hepatic CD1d-reactive T cells with or without HCV infection, most readily detectable in CHC (19,21,22). Apparently, human hepatic iNKT activity was relatively rare. Non-invariant CD1d responses were somewhat less readily detectable directly \textit{ex vivo} than \textit{in vitro} from both HCV\(^+\) and HCV-negative subjects. CD1d-specific IFN\(\gamma\) was most consistently detected compared to other cytokines tested.

**Proportion of hepatic CD1d-reactive T cells \textit{ex vivo}**

Next, we addressed the fraction of IHL capable of responding to CD1d \textit{ex vivo}. IHL were co-incubated with C1R CD1d or controls in the presence or absence of different stimuli and activation determined by FACS measurement of up-regulation of CD69 and IFN\(\gamma\) production (Figure 3). A substantial fraction of control highly-enriched iNKT line cells responded to CD1d (Figure 3A,B). As expected given their low frequency in human IHL, iNKT-specific ligand \(\alpha\text{GalCer}\) did not stimulate many IHL \textit{ex vivo} (not shown), although iNKT stimulation is well known to rapidly lead to activation of first iNKT and then NK cells (both CD69 up-regulation and IFN\(\gamma\) production), followed by other immune cells downstream (9;29–32). However, 2 co-stimuli known to be active with CD1d for at least murine iNKT (IL-12) (50) and for all types of CD1d-reactive T cells (19,21,22,33,48) (‘Total’=PMA), IL-12 and PMA, each produced comparable and substantial proportions of CD1d-responsive IHL (Figure 3A,B). IL-12 has not previously been shown to co-stimulate CD1d-specific non-invariant NKT responses, so this provides an alternative to PMA. Importantly, CD1d mAb specifically reduced the proportion of CD69\(^+\) and IFN\(\gamma\)-producing IHL, demonstrating CD1d-dependency of these responses (Figure 3A,B), as previously for IHL and other NKT cell populations (19,21,22,33,48). Therefore, a substantial fraction of
human IHL, larger than the typical proportion of antigen-specific T cells (e.g. 1–9:17), is directly CD1d-reactive \textit{ex vivo}.

\textbf{Selective hepatocyte cell surface CD1d up-regulation in active CHC without history of alcohol}

To date, only limited CD1d expression has been shown in human liver. These are at trace levels inside normal hepatocytes (26,27), increased expression by biliary epithelia in PBC (27) and in HCV infection (21), by unidentified cells adjacent to hepatic stellate cells in HCV cirrhosis (20), and on hepatic mononuclear cell surface in normal liver (22).

Figure 4 shows hepatocyte CD1d surface expression compared to both related CD1a and isotype control antibody staining \textit{ex vivo}. Uninfected livers expressed little if any hepatocyte cell surface CD1d, with at most, limited expression in ESLD amyloidosis (Figure 4). Samples with non-HCV ESLD hepatitis (fulminant HBV; acute HAV and HBV, both chronic alcohol users) also did not show detectable hepatocyte CD1d (Figure 4). However, CD1d was specifically up-regulated on most hepatocytes in simple active CHC (Figure 4). Interestingly, where alcohol was known to be involved, no significant increase in hepatocyte CD1d was detected alone or in the presence of HCV, HBV or HAV (Figure 4). Similarly, resolved HCV infection and HCV treatment responders lacked hepatocyte CD1d up-regulation (Figure 4). Results were confirmed with CD1d-specific mAb (not shown) reactive with distinct epitopes (25). This selective up-regulation of hepatocyte surface CD1d in CHC extends previous data showing increased hepatic CD1d protein expression by immunoprecipitation/western blotting (21) or immuno-histochemistry (20,21). Together with enhanced detection of CD1d-reactive T cells \textit{ex vivo} in HCV infection, this provides supportive evidence that HCV-mediated CD1d up-regulation on hepatocytes makes them a target for destruction by the large CD1d-reactive NKT population.

\textbf{Discussion}

Here we report high fractions of mostly non-invariant hepatic CD1d-reactive T cells producing IFN\(\gamma\), some IL-10, and detectable but variable levels of IL-4 and IL-13 \textit{ex vivo}, readily detected from chronic HCV-infected subjects and somewhat less frequently from other liver diseases. Furthermore, we found surface CD1d specifically up-regulated by hepatocytes in CHC. These results extend previous data on relatively Th1-biased CD1d-reactivity of \textit{in vitro} cultured human IHL (19,21), except in cirrhosis, where Th2 cytokine levels were higher (20,21), \textit{ex vivo} HCV-negative subjects (22), and on hepatic CD1d (20–22). We detected CD1d-reactivity from >50% of HCV-negative and >75% HCV+ subjects \textit{in vitro} (19,21) (Figure 1). Therefore, \textit{in vitro} culture may enhance measurement of CD1d-reactive IHL, but Th1 bias. Human resident hepatic non-invariant CD1d-reactive NKT are evidently more like rodent Th1/Th2 iNKT (5,8,9,29–32).

CD1d can be up-regulated (20,21;40,41) or down-regulated (29–32) by infection. Therefore, apparently, certain pathogens have adopted countermeasures toward anti-microbial CD1d-reactive NKT (20,21;29–32;40,41), consistent with findings of selective defects of CD1d-reactive NKT in immunodeficiencies with viral sensitivity (29–32,38). Tissue CD1d up-regulation presumably alerts local CD1d-reactive NKT of potential infection. However, this
strategy may be exploited by HCV and other infections (20,21,40,41), supported by our finding of lack of CD1d in resolved CHC. Such induced expression could be on HCV-infected or neighboring cells. Lack of CD1d in CHC with history of alcohol may reflect a further net immuno-suppressive effect over CHC alone.

Selectively increased hepatocyte cell surface CD1d expression in simple active CHC, but apparently not resolved CHC or other hepatotropic viral infections, together with enhanced detection of hepatic CD1d-reactivity, specifically implicates the CD1d:NKT axis in hepatitis C immuno-pathology. High level hepatic CD1d-reactivity has implications for therapeutic applications of NKT subsets (51,52).

Acknowledgments

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Abbreviations

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<tr>
<td>IHL</td>
<td>intrahepatic lymphocyte</td>
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<td>NKT</td>
<td>natural killer T cell</td>
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References


Figure 1. Comparison of hepatic CD1d-reactive T cells assayed directly *ex vivo* versus after *in vitro* expansion: cytokine profile of hepatic CD1d-reactive T cells *ex vivo*

Day 3 total (net) CD1d reactivity was measured as described previously (19,21,22,33,48,49). Limit of detection was 1 ng.mL$^{-1}$; modest responses were 1–10 ng.mL$^{-1}$; strong responses: >100 ng.mL$^{-1}$. Pure blood iNKT lines provided positive controls.

A. *Ex vivo* CD1d-specific and mitogen IFN$\gamma$ responses of IHL from HCV$^\pm$ subjects, compared to *in vitro*-expanded IHL.

B. % CD1d-specific IFN$\gamma$ responses of IHL *ex vivo* relative to mitogen, compared to HCV$^\pm$ donor *in vitro*-expanded IHL. There were no significant IHL responses to invariant NKT-specific ligand $\alpha$Galcer (not shown), although pure blood iNKT served as positive controls.

C. Total (net) CD1d-specific and mitogen IFN$\gamma$ responses of IHL from subjects without HCV were assayed *ex vivo* compared to matched donor *in vitro*-expanded IHL and pure iNKT controls.

D. CD1d-specific and mitogen IL-4 responses of the same matched IHL as in 1C. No CD1d-specific IL-4 was detected from these IHL.
E. CD1d-specific and mitogen IL-10 responses of the same matched IHL as in 1C.
Figure 2. Functional characterization of hepatic CD1d-reactive T cells \textit{ex vivo}

Cytokine CD1d-reactivity of representative IHL from subjects with or without HCV was measured \textit{ex vivo} and compared to pure blood positive control iNKT lines, as in Fig. 1.

A. CD1d-specificity was determined by measuring CD1d-reactivity \textit{ex vivo} in the presence of neutralizing CD1d or control mAb.

B. CD1d-specific, αGalCer iNKT, and mitogen IFNγ responses of 2 representative IHL from subjects without HCV assayed \textit{ex vivo}.
C. CD1d-specific, αGalCer iNKT, and mitogen IFNγ responses of IHL from a third representative subject assayed \textit{ex vivo}.  

D. CD1d-specific, αGalCer iNKT, and mitogen IL-4 responses of IHL from the same subject as in Fig. 2C assayed \textit{ex vivo}.  

E. Total CD1d-specific, αGalCer, and mitogen IFNγ responses of IHL from 3 representative HCV+ subjects, an HCV+ subject with documented chronic alcoholism, and 2 HCV-negative subjects assayed \textit{ex vivo} compared to iNKT lines.  

F. Summary of representative CD1d-specific IFNγ reactivity data. Subjects: 5 chronic HCV+ subjects, 5 HCV+ subjects with documented history of alcohol use, 2 chronic HBV+ subjects, 1 acute HAV+ subject, and 14 patients with other non-viral liver diseases were analyzed. iNKT controls also shown.  

G. CD1d-specific and mitogen IL-13 responses of IHL from 3 representative subjects (2/3 were IFNγ CD1d-reactive) assayed \textit{ex vivo}. 
Figure 3. Identification of IFNγ-producing hepatic CD1d-reactive T cells ex vivo

Ex vivo IHL and iNKT control lines were co-incubated with C1R CD1d in the presence or absence (‘control’) of IL-12 or PMA (‘total’ CD1d-specific). Activated cells were identified by FACS of CD69 up-regulation (A.) and production of IFNγ (B.). CD1d mAb or isotype control were included to determine specific CD1d-dependency.
Figure 4. CD1d expression by hepatocytes from HCV-infected subjects ex vivo

FACS of hepatocytes ex vivo from control and HCV+ subjects were stained with CD1a or isotype control mAb (dotted tracing) or CD1d mAb (solid curve, except normal liver, bold line). Samples were ESLD Caucasian or African American males in upper 40s–50s. CD1d+ hepatocytes in CHC shown. ESLD PSC, chronic HCV with documented chronic alcohol use, alcohol alone, HAV, and/or HBV infections had little or no detectable CD1d expression. Minimal CD1d expression by a fraction of hepatocytes in ESLD amyloidosis.
shown. Control CD1d and CD1a C1R transfectants also shown. Similar results were found with other CD1d mAb to distinct epitopes.
### Table 1

**Subject Status and Relative Hepatic IFNγ Production *ex vivo***

Subject clinical and other status and % CD1d-specific responses relative to mitogen ± SEM. EtOH: documented history of alcoholism. Unk: unknown cadaver donor.

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### Table 2

Reagents & Source

Reagents used and source.

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<td>130-054-202</td>
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