IL-17 Producing Cells and RORγt mRNA Transcriptional Factor in Cirrhotic and HCC Egyptian Patients

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Retinoic acid-related orphan receptor γT (RORγT) is the orphan nuclear receptor that regulates the development of Th17 cells and the expression of IL-17. The differentiation of Th17 cells is associated with the upregulation of RORγT mRNA, and the mechanisms regulating that process in human cells are not well understood. RORγt as transcription factor was selectively expressed in Th17 cells and is regulated by STAT3. The relationship between Th17 cells and tumor immunopathology has been controversial. Aim of the study is to evaluate Th17 cells and RORγt transcriptional factor in cirrhotic, early and advanced HCC patients. Ninety patients were studied (30 cirrhotic, 30 early stage and 30 advanced stage HCC patients). They were recruited from the National Liver Institute, Menoufia University, and subjected to full clinical examination, investigations to detect liver cirrhosis, portal vein thrombosis and tumor staging. Peripheral blood mononuclear cells (PBMCs) were stimulated with cytosim, ionomycin and monensin, and surface (CD4-PE) as well as intracellular staining for Th17 (IL-17 FITC) was performed. Analysis of cells was carried out using FACS Calibur. RORγt mRNA expression in PBMCs was measured by real time RT-PCR. Cirrhotic patients showed increased Th17% cells, without significant change in RORγt mRNA as compared to early stage HCC. Advanced stage HCC patients showed significant increase of Th17 cells% and RORγt mRNA compared to studied patients group. Positive correlation of Th17 and RORγtmRNA was found with aminotransferases and bilirubin levels while, negative with serum albumin in advanced stage patients group (P<0.001). Both markers were significantly increased with tumor size; RORγt mRNA increased with multiple tumor foci. In conclusion, Th17 cells and RORγt may be useful prognostic markers for advanced liver cirrhosis and HCC.

helper 17 cells (Th17) are lineage of T helper cells that are induced under anti-Th1/Th2 polarizing conditions and preferentially produce interleukin-17 (IL-17) (Romagnani et al., 2009). Th17 express markers such as CD26, CD161, and interleukin-4-inducible gene (Annunziato et al., 2012). IL-17-producing CD4+ T helper cell subset cells, has been described to have potent pro-inflammatory properties and play an active role in inflammation and autoimmune diseases (Park et al., 2005).

Th17 cells are enriched at mucosal site where they are thought to play a role in maintenance of immune homeostasis in response to commensal organisms and protect against pathogens that may gain entry via these surfaces (Mucida & Salek-Ardakani, 2009).

Retinoid Orphan Nuclear Receptor Gamma t (RORγt) is a Th17-specific transcription factor. It is the first transcription factor to be selectively expressed in Th17 cells (Ivanov et al., 2006) and is regulated by STAT3 (Laurence et al., 2007). It has been shown that, overexpression of RORγt promoted Th17 differentiation when Th1 and Th2 development was inhibited (Ivanov et al., 2006). Conversely, RORγt deficiency resulted in profound Th17 deficiency and was reported to protect mice from EAE. However, RORγt defect did not completely abolish Th17 differentiation or totally inhibit EAE, suggestive of additional factors involved (Yang et al., 2008).
Although Hepatitis B virus (HBV) causes chronic immune-induced liver injury and forces disease progression from mild to severe inflammation, to fibrosis, and finally to cirrhosis (Li et al., 2012). Such damage has conventionally been attributed to killing of infected hepatocytes by virus-specific cytotoxic CD8+T cells (Szabo et al., 2007). Increasing evidence, however, suggests that non-HBV-specific inflammatory infiltration into the liver is likely responsible for hepatic pathology in patients with chronic hepatitis B (CHB) (Rehermann, 2007).

Hepatocellular carcinoma (HCC) is usually derived from inflamed fibrotic and/or cirrhotic liver with intensive immune cell infiltration. Thus, the immune status may largely influence the biologic behavior of HCC (Shirabe et al., 2010). Despite improved diagnostic and treatment strategies most HCC patients are diagnosed at advanced stages and are not eligible for potential curative therapies (Liao et al., 2013).

Substantial evidence indicates that although cancer patients exhibit a generalized immunosuppressive status, the inflammatory reaction at tumor site can foster tumor growth and progression (Zhang et al., 2009). It has been reported that increased circulating regulatory T cells and IL-6 predict poor survival, while circulating NK cell activation associated with improved survival in HCC patients (Pang et al., 2011). However, the relationship between Th17 cells and tumor immunopathology has been controversial and highly depends on the context (Zou et al., 2010).

The study aimed to evaluate Th17 cells percentage and its transcriptional factor RORγt in cirrhotic and hepatocellular carcinoma patients at early and advanced stages.

Patients and Methods

Patients

Ninety patients were included suffering from HBV infection liver disease; 30 patients with cirrhosis confirmed by laboratory investigations and ultrasonography revealed surface nodularity, coarse and heterogeneous echo texture. Portal hypertension, Splenomegally. Ascitcs and Doppler flow changes were reported. Sixty HCC patients (30 early stage patients (A1–A3) stages and 30 advanced stage patients (B – C multiple foci) stages of HCC according to Barcelona-Clinic Liver Cancer (BCLC) staging system). Their ages ranged between 49 and 67 years. Cases with chronic inflammatory, autoimmune diseases, hematological, and tumors of any organ other than liver were excluded. They were recruited from outpatient clinic and inpatient Hepatology department at the National Liver Institute, Menoufia University, Menoufia Governorate, Egypt. All patients were consented after study approval of our ethical committee. They were subjected to clinical examination, laboratory investigations and abdominal ultrasonography to detect liver cirrhosis, their HBV viral load were up to 60.000, 67.000, and 83.000 units in studied groups respectively. Portal vein thrombosis and tumor staging were examined.

Materials and Methods

Th17 Cells measurement

Basic principle: Separate PMNC cultured with buffers to be stimulated to secrete IL17. Then collect them to be measured on flowcytometry to assess the percentage of Th-17 cells which a subset from T helper cells.

- Reagents
  - Two monoclonal antibodies (MAbs), one directed against human leukocyte surface marker CD4-phoerythrin (PE), and the other is anti-human IL17-fluorescein isothiocyanate (FITC eBioscience San Diego, CA).
  - Culture medium RPMI 1640 with L-Glutamine from Euroclone, supplemented with 5% autologous human serum, Ficol-paque solution (Bio-Rad medical diagnostics GmbH –Germany).
  - Monensin Solution (Golgi stop reagent): an inhibitor of intracellular protein transport leads to blockade of protein transport to Golgi Complex and accumulation of proteins in endoplasmic reticulum eBioscience (San Diego, CA) Supplied at 1000X working concentration (diluted in culture media1:1000) to reach a final concentration of 2 µM.
-CytoStim (Miltenyi Biotec GmbH, Germany (Catalog number: 130-092-173) developed for rapid and efficient stimulation of T cells by binding T cell receptor and crosslinking to MHC molecule of antigen presenting cells. Upon stimulation with CytoStim, CD4 cells start to secrete effector cytokines and up-regulate activation markers on their cell surface within a few hours

-IC Fixation Buffer (1X) (Catalog number: 00-8222) and Permeabilization buffer (10X) (Catalog number: 00-8333) used to fix and permeabilize cells prior to performing intracellular staining of cytokines (eBioscience San Diego, CA).

- Procedure

Cell culture performed through samples collected in heparinized vacutainers (Becton Dickinson, San Jose, CA). PBMCs were separated by standard Ficoll-Paque density gradient centrifugation (1800 rpm for 20 minutes) then harvested and washed twice by phosphate buffer saline (PBS) by centrifugation at 3000 rpm for 5 minutes then aspirate supernatant.

Cells re-suspended in RPMI 1640 supplemented with 5% autologous serum at a density of 10^7 cells per ml. Cells were cultured into 2 tubes one negative control where the sample is treated the same as the stimulated sample, except for 20 µl cytostim/mL cell suspension. Cells were mixed and incubated for 2hrs, then incubated with Monensin (2uM) for 4 hrs.

Flowcytometry Analysis Cells were immunostained with 10µl MABS CD4- PE, 100 µl IC fixation buffer, then 100 µl permeabilization buffers (10 x) and finally 10µl of anti-human IL-17 FITC monoclonal and analyzed in FACS Calibur using WinList software (WinList 8.0 cytometry software bar for data analysis, automated V-Comp™ compensation, 3D graphics, and validated accuracy).

Interpretation of Result as acquisition/analysis of flow data Mean Fluorescent Intensity (MFI) as dual population showing distinct negative and positive population. Measured percentage positive cells (Th-17) and mean fluorescence intensity of positive cells were compared to reveal level of expression IL 17 cytokine. (Moseley et al., 2003), (Fig. 1)

RORγt transcriptional factor

- Principle RORγt mRNA performed by Real time PCR (The Abbott m2000 Real Time Instrument)

Test is based on sample preparation, total RNA extraction using (Abbott m24sp) Reverse transcription Polymerase Chain Reaction (RT-PCR) amplification (Abbott m2000rt).

RNA extraction, Reverse transcription, Polymerase Chain Reaction (PCR) amplification of target DNA using RORγt specific complimentary primers, hybridization of the amplified products to SYBR green dye and detection of the SYBR green dye bound amplified products by colorimetric were determined.

- Procedure

Messenger RNA extraction: EDTA blood samples (stored for a short time at 4°C) were utilized for the experiments. Messenger RNA from lymphocytes was extracted after Ficoll separation. Samples were kept on ice throughout all of the procedures. Messenger RNA from 1–5 x 10^6 lymphocytes was purified with oligo dT coupled to magnetic beads according to manufacturer (DYNAL® Magnetic Beads, Invitrogen, CA, USA). After release from the magnetic particles, 100 – 500 ng mRNA was collected for the experiments.

- Quality of mRNA

To assess the quality of the mRNA amplification, the Gene Checker Kit by Invitrogen was used. This kit contains five primer sets that are designed to hybridize only to cDNA, not to genomic DNA. With this kit, mRNA or cDNA can be evaluated for the presence of large (> 6 kb) (Catalog no ( K 1370–01 Version C, Invitrogen, CA, USA).

- PCR Procedure

Twenty microliter reaction mix contained: LightCycler RT-PCR Reaction Mix SYBR Green 1x (Roche Applied Science), 5 mM MgCl2, 0.4 µl LightCycler RT-PCR Enzyme Mix, 20 ng mRNA, 0.5 µM of each primer; add H2O to a total volume of 20 µl.

The PCR conditions were as follows: reverse transcription: 55°C, 30 minutes; denaturation: 94°C, 2 minutes. Forty-five PCR cycles were run at: denaturation 94°C, 1 second, ramp time 20°C s^-1; annealing 60°C, 10 seconds, ramp time 20°C s^-1; touchdown to 55°C at 1°C/cycle; extension 72°C, 60 seconds, ramp time 1°C s^-1; melting curve: 65°C–95°C, 20°C s^-1; Fluorescence measurement FL1 (SYBR Green).

The amplified cDNA was quantified on a photometer at 260 nm. For each sample, mRNA expression level was normalized to the level of GAPDH housekeeping genes. The expression ratio was analyzed using the delta comparative cycle threshold method.
Figure 1. Flow cytometric analysis of Th17% cells in studied groups showing gating of lymphocytes (a) and % of cells in different groups 1.74% for cirrhotic, 2.99% for early HCC and 4.40% for advanced HCC.
Primers: (Metabion international AG ( Martinsried, Deutschland).

RORγt: Master regulatory transcription factor of Th17 lineage
Forward : GCT GTG ATC TTG CCC AGA ACC
Reverse CTG CCC ATC ATT GCT GTT AAT CC

GAPDH: housekeeping gene
Forward : 5'-AGC CAC ATC GCT CAG ACA C-3'
Reverse 5'-GCC CAA TAC GAC CAA ATC C-3'

(Jetten et al., 2001).

Results

Positive significant correlation between Th17 cells% and RORγt mRNA was demonstrated to (LFT) aminotransferases and bilirubin levels while, negative with albumin levels in studied patients groups (P<0.001) (Table 1).

Table 1. Pearson's correlation of Th17% and RORγt mRNA with LFT among the studied patients' groups

<table>
<thead>
<tr>
<th>LFT</th>
<th>Th17cells &amp; RORγt mRNA</th>
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<tbody>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>ALT ( IU/mL)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
</tr>
<tr>
<td>AST (IU/mL)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>Direct Bilirubin (mg/dl)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>-0.48</td>
</tr>
<tr>
<td></td>
<td>-0.44</td>
</tr>
</tbody>
</table>

P<0.05 is significant. P<0.05 is significant.

Cirrhotic patients revealed rise in Th17% cells compared to HCC early stage patients (Table 2 & 3).

Table 2. Th17 cells among patients with Cirrhosis and HCC

<table>
<thead>
<tr>
<th>Th-17 cells %</th>
<th>Early HCC Group II</th>
<th>Advanced HCC Group III</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>1.83±0.39</td>
<td>2.57±0.59</td>
<td>P1&lt;0.05</td>
</tr>
<tr>
<td>Range</td>
<td>1.14-2.52</td>
<td>2.02-4.55</td>
<td>P2&lt;0.001</td>
</tr>
</tbody>
</table>

P1=Comparison between Group I and II
P2= Comparison between Group I and III
LSD=post-hoch test for multiple comparison between groups. P<0.05 is significant.
Table 3. RORγt mRNA level among patients with Cirrhosis and HCC.

<table>
<thead>
<tr>
<th></th>
<th>Cirrhotic Group I</th>
<th>Early HCC Group II</th>
<th>Advanced HCC Group III</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>1.37±0.37</td>
<td>2.29±1.26</td>
<td>4.75±2.05</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.0-2.19</td>
<td>1.23-5.20</td>
<td>2.0-7.00</td>
<td></td>
</tr>
</tbody>
</table>

P1=Comparison between Group I and II
P2= Comparison between Group I and III
LSD=post-hoc test for multiple comparison between groups. P>0.05 is not significant (NS).

In HCC advanced stage patients both Th17 cells % and RORγt mRNA showed a significant rise compared to cirrhosis and early HCC patients groups. Both markers were significantly increased along with tumor size (Table 4).

Table 4. Comparison of Th 17 cells % and RORγt mRNA in HCC patients in relation to tumor size

<table>
<thead>
<tr>
<th>Variables</th>
<th>&lt;5 cm (Mean±SD)</th>
<th>&gt;5cm (Mean±SD)</th>
<th>Test of significance</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th 17 cells %</td>
<td>2.74±0.69</td>
<td>3.56±0.90</td>
<td>3.02*</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>RORγt mRNA IU/ml</td>
<td>2.73±1.78</td>
<td>4.72±2.02</td>
<td>3.20*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

# t-test. *Mann Whitney U test. P<0.05 is significant.

Th17 cells% were significantly increased with tumor extension. RORγt mRNA revealed significant increase in multiple tumors Foci advanced Cases (Fig. 2).

Figure 2. Comparison of Th17 cells % and RORγt mRNA among HCC patients in relation to tumor multiplicity showing increase both markers in multiple foci tumours than single ones
Receiving Operating Curve (ROC) (Fig. 3) Area under the curve (AUC) of Th17 cells % and RORγt mRNA were 0.98 and 0.96 respectively. The cut off level was 1.99% for Th17 cells% and 1.19 for RORγt mRNA. Their specificity 87%, 73% PPV was 90%, 81% were sensitive for HCC and cirrhotic respectively.

**Table 5. Comparison of Th17 cells %, RORγt mRNA for differentiation among patients with or without HCC.**

<table>
<thead>
<tr>
<th>Cutoff point</th>
<th>Area under ROC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>+ve predictive value</th>
<th>-ve predictive value</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th 17 cells%</td>
<td>≥1.99</td>
<td>0.98</td>
<td>100 %</td>
<td>87 %</td>
<td>90 %</td>
<td>100 %</td>
</tr>
<tr>
<td>RORγt mRNA IU/ml</td>
<td>≥1.19</td>
<td>0.96</td>
<td>100 %</td>
<td>73 %</td>
<td>81 %</td>
<td>100 %</td>
</tr>
</tbody>
</table>

**Figure 3.** ROC curve TH17 %( model 1), RORγt mRNA (model 2) showing high sensitivity 100 % of both markers and specificity of 87% and 70% respectively.

**Discussion**

Th17 cells play a critical role in protection against pathogens their response can be induced by virus antigens (Rowan *et al.*, 2008). IL-17 produced by Th17 cells serves as a chemo-attractant for neutrophils to sites of infection and inflammation.

In this study, cirrhotic patients showed a significant rise in Th17% cells not seen for RORγt mRNA compared to early HCC group. Positive correlation between Th17% and similar for RORγt mRNA with aminotransferases mainly ALT levels, which often serves as a marker of hepatic injury, total and direct bilirubin in studied patients groups ($P<0.001$) while, a negative correlation with serum albumin were revealed. These results suggest that Th17%
reflects both the hepatic synthetic function and the inflammatory activity/or injury.

These results agreed with Xue-Song et al., (2012) who reported that patients with chronic HBV infection favored Th17 cell differentiation, accompanied by a higher proportion of peripheral Th17 cells. In addition Li et al., (2012) and Sun et al., (2012) reported that, the frequency of Th17 in the diseased liver correlates with liver fibrosis in patients with chronic hepatitis B.

Zhang et al., (2010) reported that Th17 are highly enriched in both peripheral blood and liver of chronic hepatitis B patients and exhibit a potential to exacerbate liver damage.

Hammerich et al., (2011) reported a close correlation between liver infiltrating as well as circulating Th17 cells and the amount of liver damage. They also reported that, a shift from Th1 to Th17 seems to be potentially disadvantageous for the patient in terms of antiviral defense and liver disease progression. Since stronger Th17 responses are associated with higher viral plasma load, increased levels of serum transaminases.

In HCC patients group, the study gained results showed that, both Th17 cells % and RORγt mRNA showed a significant rise in advanced HCC patients in comparison to cirrhosis and early HCC patients. In addition, it was found that both markers were significantly increased with increase of tumor size. Th17 cells were significantly not increased with tumor extension. While RORγt mRNA, showed significant increase in multiple tumors foci cases. However, both Th17 cells and RORγt mRNA rise was not significant in relation to vascular invasion and distal metastasis.

Using ROC analysis, the area under the curve (AUC) for Th17 cells % and RORγt mRNA were 0.98 and 0.96 respectively compared to the minimal acceptable area (0.7) of a parameter to be considered as a good diagnostic tool (Swets, 1988), An assumption that both Th17cells% and RORγt mRNA are highly acceptable markers for hepatocellular carcinoma. Although the cut off level for the best diagnostic value needs to be clarified with a large sample study, our results showed that the cut off level was 1.99% for Th17 cells% and 1.19 unit for RORγt mRNA at these levels the test showed the highest sensitivity and specificity.

The up regulation of Th17 cells in hepatocellular carcinoma patients and its positive correlation with the stage of the disease were reported by several studies. Greten et al., (2012) showed that Th17 cells in peripheral blood from HCC patients were at higher frequencies than patients with other liver diseases. Wang et al., (2010) reported that the level of Th17 cells in peripheral blood of patients with advanced stage of HCC (III-IV) was significantly higher than that in early stage (I-II, P = 0.0008) of hepatocellular carcinoma. They also reported that the increased level of Th17 cells in HCC patients showed a positive correlation with the tumor size, portal vein thrombosis and metastasis.

Th17 cells were suggested as a prognostic marker for HCC Liao et al., (2011) revealed that, production of IL-6 and TNF-α cytokines can create inflammatory cytokine milieu that promotes the expansion of human Th17 cells. They have shown that high expression of IL-17 in HCC was associated with worse clinical outcome after resection (poor survival and early recurrence).

An increase in Th17 cells has been detected in peripheral blood, tumor microenvironment and tumor-draining lymph nodes of several different human and mouse tumor types (Su et al., 2010). The association between Th17 cells % and the worse clinical outcome of HCC is attributed to IL-17 that can promote tumor growth by angiogenesis. Therefore variables such as tumor- initiating agents, a pre-existing inflammatory environment and the immune competence of
the host may have direct effect upon T-helper subset cells and may profoundly reshape the generation and function of Th17 cells in cancer patients (Wilke et al., 2011). Finally, Cirrhotic had high Th17 cells so as early stage HCC patients higher in advanced cases. RORγt mRNA was raised significantly only in advanced cases. Th17% and RORγt mRNA increase was associated with the stage and tumor size of hepatocellular carcinoma. Th17 cells increased with tumor extension while RORγt mRNA in multiple tumor foci cases.

References


