Hepatitis Monthly 2008; 8(3): 173-177

ORIGINAL ARTICLE

Assessment of Treg Cells $CD4^+CD25^+$ in Chronic Cirrhotic Liver Disease and Hepatocellular Carcinoma Egyptian Patients

Maha A. El Bassuoni [1*], Mones A. Obada [1], Tarek Korah [2], Sawsan El Sayed [3]

[1] Department of Clinical Pathology, Faculty of Medicine, Menoufiyia University, Cairo, Egypt
[2] Department of Internal Medicine, Menoufiyia University, Cairo, Egypt
[3] Department of Tropical Medicine, Menoufiyia University, Cairo, Egypt

Background and Aims: **Dysfunction of the host immune system in cancer patients can be due to a number of factors, including secretion of tumor-derived immunosuppressive factors or induction of immune tolerance against tumor-specific antigens. Several studies suggest that suppression of tumor-associated antigen reactivates lymphocytes by $CD4^+$ $CD25^+$ regulatory T (T_{reg}) cells. This study was designed to evaluate whether $CD4^+CD25^+$ T_{reg} cells in chronic liver disease and hepatocellular carcinoma (HCC) patients exhibit an expanded T_{reg} pool and to correlate it with liver tumor markers and grading.**

Methods: **Blood samples were collected from 20 patients with cirrhotic liver disease (CLD), 15 HCC patients and 10 healthy control subjects. Alpha feto-protein (AFP), HBV and HCV antibodies were detected by EIA. HCV was confirmed by immunoblotting and RT-PCR. To evaluate HCC grading, abdominal ultrasound guided liver biopsy was done. Patients were categorized into moderately differentiated (grade II) and poorly differentiated (grade III) groups. Cytometric analysis of $CD4^+CD25^+$ T_{reg} cells in PBMCs was performed using anti-CD3, anti-CD4, anti-CD25, anti-CD45RA, and IgG-isotype control (FITC and PE).**

Results: **Both CLD and HCC groups were 80% positive for HCV while only 20% of CLD and 11% of HCC patients were positive for HBV. The mean percentage of $CD4^+CD25^+$T cell population demonstrated a highly significant increase in comparing HCV to HCC patients [2.47±0.66 vs. 8.96±1.38 ($P<0.001$)] and when comparing both group to controls [1.15±0.5 ($P<0.01$)]. Nine HCC patients were in grade II while 6/15 were in grade III. Their mean $CD4^+CD25^+$ T cells percentage was 9.12±1.52 and 8.73±1.33, respectively. A negative correlation was found between mean $CD4^+CD25^+$ T cells percentage and AFP serum level in HCC patients ($r=-0.923$) while T_{reg} cells with patients tumor grades (II and III) ($r=0.474$ and 0.582, respectively). CLD showed a significant correlation with AFP level ($r= 0.962$).**

Conclusions: **Tumor specific T_{reg} cells may limit the efficacy of anti-tumor response. T_{reg} cells correlate properly with the unique marker AFP and with tumor grades. Better understanding of the underlying mechanism of T_{reg} regulation or of the strategy for controlling T_{reg} cells may lead to effective HCV immunotherapy and enhancing immunity against cancer.**

Keywords: **$CD4^+CD25^+$ T_{reg} Cell, HCV, Hepatocellular Carcinoma, Liver Cirrhosis**

Introduction

The emergence of a tumor results from the disruption of cell growth regulation as well as from failure of the host to provoke a sufficient immunological anti-tumor response [(1)]. Indeed, most cancer patients do not develop a satisfactory immunological antitumor response, implicating the existence of tumor-specific immune evasion strategies [(2, 3)]. Dysfunction of the host immune system in cancer patients can be due to a number of

**** Correspondence:***
Maha A. El Bassuoni, M.D., Department of Clinical Pathology, 11 el Mashad St., Heliopolis, Cairo, Egypt.

Tel: *+20 482228303*
Fax: *+20 225328286*

E-mail: *mabassuoni@gmail.com*

Received: *22 May 2008* ***Revised:*** *7 Jul 2008*
Accepted: *24 Jul 2008*

Hep Mon 2008; 8 (3): 173-177

factors, including the secretion of tumor-derived immunosuppressive factors, such as IL-10 and TGF-β [4] or the induction of immune tolerance against tumor-specific [5].

In vitro studies demonstrate that $CD4^+CD25^+$ T cell population appears to be a homogeneous population of suppressors that do not contain memory or activated T cells [6]. $CD4^+CD25^+$ T cell population present in normal mice is a potent inhibitor of polyclonal T cell activation. Suppression is mediated by a cytokine-independent, cell contact-dependent mechanism that requires activation of the $CD4^+CD25^+$ cells via the TCR [7]. It is well known that TILs and, to a lesser extent, PBLs from patients with advanced-stage cancer have a poor immune response [8]. This tumor-induced immunosuppression includes diminished responses to recall antigens, decreased proliferative T-cell responses and loss of cytokine production, and defective signal transduction in T cells and natural killer cells [9]. There is also evidence for increased apoptosis among $CD8^+$ T cells in PBLs from cancer patients and mice with experimental tumors.

Several studies suggest that suppression of tumor-associated antigen reactivates lymphocytes by $CD4^+CD25^+$ regulatory T (T_{reg}) cells [10] in cancer patients and that elevation of T_{regs} may down-regulate tumor-specific immunity. In healthy humans, this population accounts for 5-10% of peripheral $CD4^+$ T cells. T_{regs}, characterized by coexpression of CD4 and CD25 markers, are thought to be a functionally unique population of T cells and function to maintain immune homeostasis [1]. Of note, T_{regs} can inhibit the immune response mediated by $CD4^+CD25^-$ and $CD8^+$ T cells because it has been reported that T_{regs} play an important role in preventing allograft rejection, graft-*versus*-host disease, and autoimmune disease. The observation that T_{regs}-depleted mice develop a broad range of autoimmune diseases suggests that this T cell subset plays a crucial role for the control of T cell-mediated immunity [11].

In addition, patients and experimental models with cancer showed that T_{regs} down-regulates the activity of effector function against tumors, resulting in T-cell dysfunction in cancer-bearing hosts. These observations have led to the hypothesis that tumor-bearing hosts with advanced cancers have an increased population of T_{regs}, which might inhibit the tumor-specific T-cell response. In fact, an increased population of T_{regs} has been reported in patients with ovarian cancer, lung cancer, and breast cancer [12, 13]. Regulatory T cells that recognize organ-specific antigens are attracted to the involved organ, are re-stimulated by their target antigen, and mediate suppression. Suppression may be mediated by the production of suppressor cytokines in the target organ with bystander suppression of the effector cells or suppression may be mediated by direct cell-cell contact of the suppressors with the effectors [14].

Notably, removal of regulatory T cells can also evoke effective antitumor immunity in mice injected with syngeneic cancer cells. Therefore, depletion of T_{regs} may enhance the anti-tumor immunity of host, but the pathogenic and mechanistic relationship between cancer and T_{regs} is still unclear [15]. The aim of this study was to evaluate whether $CD4^+CD25^+$ T_{reg} cells in HCV chronic liver and HCC patients exhibit an expanded T_{reg} pool and to correlate it with liver diagnostic investigations and tumor grading.

Materials and Methods

Blood samples were collected from 20 patients with chronic hepatitis C (mean age: 50±9.9 years) and 15 patients with HCC (mean age: 60±8.3 years). Alpha-feto protein and HCV positive antibodies were detected by EIA and immunoblotting techniques confirmed by RT-PCR. The HCC group grades were categorized into grade I, II, III. We consecutively recruited all asymptomatic patients who showed an increase of 1.5 times or higher in aminotransferases levels, as recorded at least twice during 6 or more months, and those who had no major contraindication to liver biopsy. The histologic grading was classified according to the criteria of the METAVIR Cooperative Study Group they subclassified into 3 grades I, II, III. Ten blood samples from normal control subjects (mean age: 37±7.4 years), who were negative for anti-HCV antibodies were also included in this study.

Laboratory investigation

All studied groups were subjected to: 1) liver function tests: ALT, AST, GGT, total protein, alkaline phosphatase, direct and indirect bilirubin (CX5 auto-analyser); 2) alpha-feto protein tumor marker: Enzyme Immune Assay (EIA) (Can-Ag kit); and 3) evaluation of T_{reg} in peripheral blood mononuclear cells (PBMCs) from an adequate number of HCC patients and correlating them with tumor burden.

Flow cytometric analysis

To determine the T_{reg} cell phenotype percentage, three- and four-color flow cytometry of whole blood

or isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells was performed using the following antibodies: anti-CD4; anti-CD11a; anti-CD25; anti-CD45RA; and IgG1-isotype control (either FITC-, PE-, peridin chlorophyll protein-; all purchased from BD PharMingen). For whole blood staining, 50 µl of whole blood was incubated with appropriate amounts of fluorochrome-labeled antibodies in the dark at room temperature for 30 min, washed once, and analyzed. To compare the phenotype of recently *in vitro* activated T cells with the $CD4^+CD25^+$ population, 1 ml of whole blood was stimulated with 10 ng/ml PMA (Sigma) and 0.5 µg/ml Ionomycin (Sigma) for 48 h before FACS analysis. Flow cytometry was performed on a Becton Dickinson FACS Calibur and Cell Quest software was used for analysis.

Statistical analysis

Data was statistically analyzed using SPSS (Statistical Package for Social Science) program version 11.0. Chi-square test was done for qualitative variable analysis and $P<0.05$ was considered significant. Fischer's exact test for 2×2 tables was used when expected cell count of more than 25% of cases was less than 5 and $P<0.05$ was considered significant.

Student's *t*-test was done for normally distributed quantitative variables to measure mean and standard deviation and $P<0.05$ was considered significant. ANOVA test was done to compare three variables: one qualitative variable and the other two were quantitative variables of normally distributed variables and $P<0.05$ was considered significant to detect mean and standard deviation where post hoc tests were done to detect the relationship between variables within groups Mann-Whitney test was done for quantitative variables which were not distributed normally and $P<0.05$ was considered significant.

Results

Both CLD and HCC groups were 80% positive for HCV while only 20% of CLD and 11% of HCC patients were positive for HBV (Table 1). The mean percentage of $CD4^+CD25^+$ T cell population demonstrated a highly significant increase in comparing HCV to HCC patients [2.47±0.66 *vs.* 8.96±1.38 ($P<0.001$)] and when comparing both group to controls [1.15±0.5 ($P<0.01$)].Nine HCC patients were in grade II while 6 were in grade III. Their mean $CD4^+CD25^+$ T cells percentage was 9.12±1.52 and 8.73±1.33, respectively. A negative correlation were found between mean $CD4^+CD25^+$ T cells percentage and AFP serum level in HCC patients group (r=-0.923) while T_{regs} with patients tumor grades (II and III) (r=0.474 & 0.582, respectively). CLD patients showed a significant correlation with AFP level (r= 0.962).

Table 1. Studied variables between HCC, CLD and control groups.

	Groups			P value
	HCC group	CLD group	Control group	
Age (yr)	60 ± 8.3	50 ± 9.9	37 ± 7.4	< 0.01
Sex (M:F)	9:6	14:6	7:3	NS
HCV (+/-)	12/4	18/4	3/7	NS
HBsAg (+/-)	3/12	2/18	0/10	NS
AFP	1028.1 ± 709.2	8.3 ± 3.16	3.35 ± 1.59	< 0.01
ALT	123.3 ± 88.8	55.4 ± 26.9	13.35 ± 1.59	< 0.01
AST	177.1 ± 186.5	61.4 ± 19.7	21.4 ± 8.68	< 0.01
GGT	180.1 ± 166.6	72.7 ± 14.25	21.6 ± 4.74	NS
Total protein	6.6 ± 0.95	6.9 ± 0.57	7.46 ± 0.41	NS
Total bilirubin	1.29 ± 0.45	1.98 ± 0.74	0.68 ± 0.14	< 0.05

Discussion

Since the function of T_{reg} cells in solid tumors has sparked interest, the present study investigated the evidence related to the prevalence of T_{regs} in HCV and HCC. We showed a significant increase in the population of $CD4^+/CD25^+$ T cells in the peripheral blood of patients with HCV and HCC in comparison with healthy individuals. Ichihara *et al.* [16] reported that there is no clear evidence for the mechanism of induction of T_{regs} in cancer-bearing hosts. There are several possibilities, including specific expansion of T_{regs} induced by cancer-derived factors, or physiological defense phenomena against the continuous inflammation induced by cancer. Moreover, Somasundaram *et al.* [17] added that, so far, little is known about the antigen-specificity of human T_{reg} cells. Ligand-specific activation and cell-to-cell contact are required for T_{reg} cells to exert suppressive activity, suggesting that the presence of tumor-specific T_{reg} cells at tumor sites may have profound effects on the inhibition of T-cell responses against cancer. In a second study, a $CD4^+/CD25^+$ T_{reg}-cell line was established from a patient with colorectal carcinoma. This T-cell line was tumor-cell dependent in its growth but did not lyse autologous

tumor cells and suppressed proliferative responses of allogeneic lymphocytes and autologous CTLs as well as the induction of CTLs from autologous PBMCs. These effects were mediated by TGF-β and did not require cell-to-cell contact, which would be in line with induced regulatory capacity. Clearly, further work is needed to understand how the enrichment of T_{reg} cells in cancer patients occurs and if accumulation or preferential induction of clonal, oligoclonal, or polyclonal tumor-specific T_{reg} cells plays a role during tumor progression.

Murine models have established that selective elimination of T_{reg} cells alone or in combination with other treatment options might induce regression of already established tumors. First pilot studies have been initiated in cancer patients to selectively eliminate T_{reg} cells. A promising and specific approach might be targeting of CD25 on the surface of T_{reg} cells [16]. Danull *et al.* [18] used IL-2 diphtheria toxin conjugate DAB (389) IL-2 (denileukin diftitox) to selectively eliminate CD25-expressing T_{reg} cells from the PBMCs of cancer patients without inducing toxicity on other cells that expressed CD25 at only intermediate to low levels. This first clinical study specifically eliminating T_{reg} cells has shown promising results that need to be further evaluated. Alisa *et al.* reported that identification and characterization of subsets of $CD4^+$ T cells that recognize an epitope within the AFP sequence (AFP_{46-55}) and tumor antigens may contain epitopes which activate the expansion of inducible regulatory T cells, leading to evasion of tumor control.

However, in our study T_{reg} cells which are a subset of T cells revealed a different behavior. A negative correlation was found between mean $CD4^+CD25^+$ T cells percentage and AFP serum level in HCC patients group (r= -0.999). While CLD patients group showed a significant correlation with AFP level (r= 0.770). These data explain of T_{regs} that in CLD patients group might add to the immune inhibitory that exit with the approach of tumor development and carcinogenesis. The present study leads to the observations and provide additional insight into the regulatory mechanisms responsible for immunosuppression in human cancer, which facilitates local tumor growth and metastasis. Metastasis often represents the fatal step during the course of malignancy; T_{regs} were correlated significantly with patients' tumor grades (II and III) (r=0.474, 0.582). They were the tumor grades which were enhanced by the suppression of immunosurveillance mechanisms. Moreover, T_{regs} may also negatively impact the effectiveness of immunotherapies.

Current attempts at immunotherapy for cancer, including cancer vaccination or adoptive transfer of T cells, remain limited in their effect on the regression of established tumors. Even if the effective CTLs are transferred adoptively to the patients or tumor-specific CTLs are generated by tumor vaccination, there are several mechanisms by which tumor cells can escape from tumor-specific T-cell surveillance in the tumor microenvironment, as described above [19]. The presence of factors such as, oxidative metabolites, or immunosuppressive cytokines can be predicted to rapidly shut off the effector functions of CTLs. Here, the increased population of T_{regs} could be an additional problem to be resolved in immunotherapy for cancer. A better understanding of the underlying mechanism of T_{reg} regulation or of the strategy for controlling T_{regs} may lead to more effective immunotherapy for cancer [16].

Wolf *et al.* [15] in their study mention that the depletion of T_{regs} may become a successful anticancer strategy. In a mouse model, the efficacy of a therapeutic vaccine against melanoma was substantially improved by depletion of T_{regs}. They concluded that manipulation of T_{regs} in terms of their frequency and functional activity should be added to the therapeutic armentarium for enhancing tumor immunity in humans. Taken together, these data have established the concept of increased T_{reg} cells in solid tumors. An important aspect of future studies would be to clarify and describe an immune therapy by deleting T_{reg} cells as a porter of immune inhibition. However, further studies are needed to validate more specific markers as well as more sophisticated and standardized functional assays.

Conclusions

Tumor specific T_{reg} cells may limit the efficacy of anti-tumor response. T_{reg} cells correlate properly with the unique marker AFP and with tumor grades. Better understanding of the underlying mechanism of T_{reg} regulation or of the strategy for controlling T_{regs} may lead to more effective immunotherapy for HCV and for enhancing immunity against cancer.

References

1. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4 (+) CD25 (+) T cells with regulatory properties from human blood. *J Exp Med* 2001; **193**: 1303-10.
2. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J,

Enk AH. Identification and functional characterization of human CD4 (+) CD25 (+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; **193**: 1285-94.
3. Ng WF, Duggan PJ, Ponchel F, *et al*. Human CD4 (+) CD25 (+) cells: a naturally occurring population of regulatory T cells. *Blood* 2001; **98**: 2736-44.
4. Elgert KD, Alleva DG, Mullins DW. Tumor-induced immune dysfunction: the macrophage connection. *J Leukoc Biol* 1998; **64**: 275-90.
5. Staveley-O'Carroll K, Sotomayor E, Montgomery J, *et al*. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A* 1998; **95**: 1178-83.
6. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* 2000; **164**: 183-90.
7. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**: 287-96.
8. Miescher S, Whiteside TL, Carrel S, von Fliedner V. Functional properties of tumor-infiltrating and blood lymphocytes in patients with solid tumors: effects of tumor cells and their supernatants on proliferative responses of lymphocytes. *J Immunol* 1986; **136**: 1899-907.
9. Kiessling R, Wasserman K, Horiguchi S, *et al*. Tumor-induced immune dysfunction. *Cancer Immunol Immunother* 1999; **48**: 353-62.
10. Cao M, Cabrera R, Xu Y, *et al*. Hepatocellular carcinoma cell supernatants increase expansion and function of CD4 (+) CD25 (+) regulatory T cells. *Lab Invest* 2007; **87**: 582-90.
11. Sakaguchi S, Sakaguchi N, Shimizu J, *et al*. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001; **182**: 18-32.
12. Woo EY, Yeh H, Chu CS, *et al*. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 2002; **168**: 4272-6.
13. Liyanage UK, Moore TT, Joo HG, *et al*. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 2002; **169**: 2756-61.
14. Frasca L, Carmichael P, Lechler R, Lombardi G. Anergic T cells effect linked suppression. *Eur J Immunol* 1997; **27**: 3191-7.
15. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 2003; **9**: 606-12.
16. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 2003; **9**: 4404-8.
17. Somasundaram R, Jacob L, Swoboda R, *et al*. Inhibition of cytolytic T lymphocyte proliferation by autologous CD4+/CD25+ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-beta. *Cancer Res* 2002; **62**: 5267-72.
18. Dannull J, Cerny T, Ackermann DK, Groettrup M. Current Status of Dendritic Cell-Based Tumor Vaccination. *Onkologie* 2000; **23**: 544-551.
19. Kono K, Takahashi A, Ichihara F, *et al*. Prognostic significance of adoptive immunotherapy with tumor-associated lymphocytes in patients with advanced gastric cancer: a randomized trial. *Clin Cancer Res* 2002; **8**: 1767-71.