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AASLD Basic Research Workshop Regulatory T Cells in Tolerance and Immunity to the Liver

**Sunday, November 4, 2007
John B. Hynes Convention Center, Ballroom B**

**Course Directors:
Kyong-Mi Chang, MD and Ethan M. Shevach, MD**

Description

Regulatory T cells (Tregs) play a key regulatory role in immune tolerance to self and non-self. With the rapidly increasing knowledge about the nature and role of Tregs in health and disease (e.g. autoimmunity, transplant tolerance, tumor tolerance and pathogen-specific immunity), this workshop will provide the current knowledge about regulatory T cells and their potential role in liver disease pathogenesis and therapeutics.

Goals and Objectives

- Gain knowledge in the evolving concepts about regulatory T cells and their role in immune regulation in health and disease
- Learn about the ongoing research on how regulatory T cells may contribute to pathogenesis of liver disease
- Learn about potential therapeutic application involving regulatory T cells in liver disease

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Regulatory T Cells in Tolerance and Immunity to the Liver

8:00 – 8:25am Introduction to Tregs
Ethan M. Shevach, MD

I. Tregs in Autoimmunity and Transplant Tolerance

8:25 – 8:50am Transplant Tolerance and Regulatory T cells
Wayne W. Hancock, MBBS, PhD

8:50 – 9:15am Autoimmune Liver Disease and Tregs
Eric M. Gershwin, MD

9:15 – 9:40am Treg Therapeutic Applications and Manipulations with Regulatory T Cells
Carl June, MD

9:40 – 10:00am Break

II. Tregs in Pathogen-specific and Tumor-specific Immunity

10:00 – 10:25am Tregs in Viral Hepatitis
Kyong-Mi Chang, MD

10:25 – 10:50am CD8+ T cells as Regulators of Liver Injury in Chronic HCV
Margaret J. Koziel, MD

10:50 – 11:15am Tregs in Liver Cancer
Drew M. Pardoll, MD, PhD

11:15 – 11:40am Panel Discussion and Wrap-up

Introduction to Tregs

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Studies over the past 10 years have shown that thymic-derived CD4⁺Foxp3⁺ T regulatory (Treg) cells are potent inhibitors of the development of autoimmune disease *in vivo* and also suppress the activation of CD4⁺ and CD8⁺ T cells *in vitro*. Nevertheless, the cellular target(s) and molecular basis for their suppressive effects have remained elusive. It is also apparent that major differences exist between the function of Foxp3⁺ Treg *in vivo* and *in vitro*. Most importantly, Treg are anergic *in vitro* to TCR stimulation, but proliferate in a manner indistinguishable from conventional T cells *in vivo*. A major problem in the analysis of the function of Foxp3⁺ Treg is that it is difficult to obtain large numbers of antigen-specific cells for use *in vivo*. We have demonstrated that Foxp3 expression can be rapidly induced in naïve T cells by TCR stimulation in the presence of TGF β and IL-2. TGF β -Treg have most of the phenotypic properties of thymic-derived Treg, as they are anergic/suppressive *in vitro* and maintain expression of Foxp3 both *in vitro* and *in vivo*. They can also protect Scurfy mice for at least 21 days. Antigen-specific TGF β -Treg can prevent the expansion and differentiation (Th1, Th17) of conventional T cells in immunocompetent mice following immunization with cognate peptide in CFA. TGF β -Treg specific for a gastric autoantigen can prevent the expansion of autoreactive effectors and the development of autoimmune gastritis by acting on dendritic cells (DC). DC exposed to TGF β -Treg *in vivo* were reduced in their capacity to stimulate proliferation and cytokine production by gastric antigen-specific T cells. A marked reduction in antigen presenting capacity was also seen when DC were exposed to TGF β -Treg *in vitro*. Activated thymic-derived CD4⁺Foxp3⁺ cells can induce Foxp3 expression and Treg function in naïve T cells in a TGF β -dependent manner and these TGF β -Treg can propagate their phenotype to fresh T cells by a pathway resembling infectious tolerance. We have also analyzed in depth the regulation of Foxp3 expression in human CD4⁺ T cells. Some studies have suggested that TCR stimulation of human CD4⁺Foxp3⁻ cells results in the induction of transient expression of Foxp3, but that the induced cells lack a regulatory phenotype. We have shown that TCR stimulation alone was insufficient to induce Foxp3 expression in human T cells in the absence of TGF β , while high levels of Foxp3 expression could be induced in the presence of TGF β . Although Foxp3 expression was stable, the TGF β -induced Foxp3⁺ T cells were neither anergic nor suppressive and produced high levels of effector cytokines. These results suggest that even high levels of Foxp3 expression are insufficient to define a human CD4⁺ T cells as a T regulatory cell. We have developed a novel assay in which the responder T cells were mouse CD4⁺CD25⁻ and the suppressor population was FACS sorted human preactivated CD4⁺FOXP3⁺CD25^{hi} cells. We were able to demonstrate that suppression in this model required a physical interaction between the human regulatory cells mediated by CD11a on the human cells and CD54 on the mouse responders. Surprisingly, the human regulatory T cells targeted the mouse antigen presenting cells and not the responder T cells.

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Transplant Tolerance and Regulatory T cells

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This presentation highlights how recognition of epigenetic mechanisms, of which two are to be discussed, is important for understanding the functions of Tregs and provides pharmacologic tools for their therapeutic manipulation.

The success of organ transplant (Tx) programs comes with a high price, given the nephrotoxicity, cardiovascular disease, diabetes and hyperlipidemia associated with current immunosuppression, the significant incidence of post-transplant lymphoproliferative disorders, and the high rates of graft loss from chronic rejection. These shortcomings continue to stimulate efforts to achieve clinical tolerance induction, or at least long-term allograft function despite minimal immunosuppression. Autoimmune and transplant models show the importance of naturally occurring CD4⁺ CD25⁺ Foxp3⁺ Tregs in limiting autoreactive and alloreactive immunity. Moreover, once Tregs are induced in Tx recipients by costimulation blockade, immunosuppression or other strategies, they can be adoptively transferred to naïve hosts and exert beneficial therapeutic effects. However, regardless of the numbers of cells transferred into immunocompetent hosts, therapy with Tregs alone does not appear to control alloresponses to fully MHC-disparate vascularized allografts. These considerations lead to concern as to whether the presence of Foxp3⁺ Tregs alone is sufficient to indicate the functional competency of cells to be adoptively transferred, or if adjunctive therapies might enhance their suppressive properties? In addition, in contrast to a general restriction of Foxp3 expression to a subset of CD4 T cells with suppressive functions, transient induction of Foxp3 now appears to be a general feature of human T cell activation, including by non-Tregs. Moreover, Foxp3 induction and transient expression by human effector T cells (T-eff) does not affect cytokine production or cell proliferation, and, unlike in the mouse, ectopic expression of Foxp3 is insufficient to make human CD4⁺ T cells become potent Tregs. Hence, at least for human cells, detection of Foxp3 alone in the context of active immune responses is not a reliable marker of the presence of Tregs, and leading to the question of what else do Tregs need for full function, besides Foxp3 expression?

Overview of epigenetic regulation

Chromatin organization involves DNA wound around histone octamers that form nucleosomes and are in turn folded into higher ordered structures. Core histones have N-terminal tails extending from compact nucleosomal core particles, and deacetylation of epsilon-acetyl-lysine residues in these tails affects histone-DNA and histone-non-histone protein interactions. Acetyl groups are added to histone tails by histone acetyltransferases (HATs), comprised of 3 super-families: GNAT (Gcn5-related N-acetyltransferase), MYST (with Myst1/MOF, Myst2/HBO1, Myst3/MOZ, Myst4/MORF and Tip60 as members) and p300/CBP. Acetyl groups are removed by histone deacetylases (HDACs); the 18 known HDACs are classified as class I HDACs (HDAC1, HDAC2, HDAC3, HDAC8 and HDAC11) that are mainly located in the nucleus; class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10) that can shuttle between the nucleus and the cytoplasm; and class III HDACs (SIRT1-7) located in various organelles.

Histone acetylation decreases the net basic charge and generally promotes a permissive remodeling, whereas histone deacetylation promotes a repressive chromatin state, though there are exceptions. Lastly, these same HAT and HDAC molecules can affect the functions of non-histone proteins, including transcription factors and other proteins, typically promoting protein stability by preventing ubiquitination and subsequent proteasomal degradation, but acetylation can also increase DNA binding affinity and protein-protein interactions.

Epigenetic regulation of Foxp3

(i) Acetylation - We propose that the functions of Foxp3 are promoted by acetylation through the action of one or more HATs, and are diminished through Foxp3 deacetylation by one or more HDACs, though additional roles for HDACs may be applicable. A role for acetylation is supported by our mutagenesis studies showing that Foxp3 acetylation is required for optimal Treg function, and by our findings that small molecule HAT inhibitors decrease Treg suppressive function, whereas HDAC inhibitors enhance both Treg production and suppressive function. Class II HDACs can regulate transcription by recruiting an enzymatically active SMRT/N-CoR-class I HDAC complex, and the deacetylase domain of HDAC9, for example, can be removed leaving HDAC9 still able to recruit a class I HDAC. Consistent with this, we have found that mice with a targeted deletion of a class I HDAC, HDAC2, have significantly increased numbers of CD4+CD25+ and CD4+Foxp3+ T cells, and that HDAC2^{-/-} Foxp3+ Tregs were twice as potent as WT Tregs in suppressing Teff proliferation, indicating that like class II HDACs, class I HDACs contribute to control of Foxp3+ Treg functions. Moreover, HDACi therapy does not further enhance HDAC9^{-/-} Treg suppressive function over that seen using baseline HDAC9^{-/-} Tregs, suggesting that interruption of either HDAC2 or HDAC9 is sufficient to enhance Treg function. Hence, HDAC9 functions as a tissue-specific deacetylase in Tregs, providing a key on/off switch that functions to assemble large macromolecular complexes with regulatory functions, including additional class I HDACs, HATs, DNMT and SMRT/N-CoR molecules.

Characterization of the acetylation sites central to Foxp3 function will allow generation of antibodies recognizing relevant peptide sequences containing key acetylated lysines vs. deacetylated lysines. Such Abs may allow detection and discrimination of functionally competent Foxp3+ Treg cells in blood and tissues. The feasibility of generating Abs against specific acetylated peptide sequences is demonstrated by the availability of commercial Abs to p53, E2F, PCAF, importin, GATA1, BRCA2, and Rb. Ultimately, knowledge of Foxp3 phosphorylation, nuclear translocation, acetylation and co-association with some HDACs but not others may provide the means for re-energizing the field of immunologic monitoring through the detection of fully competent vs. resting or inactive Foxp3+ Treg cells.

(ii) Methylation - Methylation of cytosine in cytosine-phosphate diester-guanine (CpG) islands, which occurs at high density at silenced genes, leads to recruitment of HDACs deacetylases, histone methyltransferase, and chromatin remodeling complexes that promote a closed chromatin conformation. Compared to the extensive cancer literature, little is known concerning DNA methylation and immune functions, though methylation of the IL-2 promoter regulates IL-2 production, with the IL-2 promoter being heavily methylated in anergic murine T cells but demethylated in T cells receiving TCR signals and CD28 costimulation. There is now evidence of complete demethylation of CpG motifs, as well as histone modifications, in the Foxp3 locus of murine Tregs but not non-Tregs, whereas developing Foxp3+ thymocytes and Tregs induced by TGF- β in vitro had only incomplete demethylation, despite high Foxp3 expression. In contrast to natural Tregs, TGF- β -induced Foxp3+ murine Tregs lost both Foxp3 expression and suppressive activity upon restimulation in the absence of TGF- β , suggesting that Foxp3 expression must be stabilized by epigenetic modification to allow development of a permanent

suppressor cell lineage. Whether cytokines such as TGF- β enhance activation-induced FOXP3 expression, or promote the recruitment of molecular complexes required for full suppressive activity, is currently under investigation. As with acetylation, consideration of methylation have both therapeutic and possible diagnostic significance. Experimentally, we have found that DNA methyltransferase inhibitors (DNMTi) can induce allograft tolerance through Treg dependent mechanisms, and that DNMTi use directly enhances Treg suppressive function. In addition, analysis of the extent of Foxp3 methylation/demethylation provides an unambiguous tool to detect Tregs in situ and to interpret their likely functional competency.

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Autoimmune Liver Disease and Tregs

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Abstract

Despite the ability of the liver to remain tolerant to foreign substances and bacterial products, and its participation in oral tolerance [1, 2], loss of tolerance in the form of autoimmunity is still observed in the liver. Regulatory T cells play critical roles in peripheral tolerance to resident microorganisms as well as autoantigens [3, 4]. Examples of regulatory T cells include forkhead box P3 (FoxP3) expressing T cells, natural killer cells, $\gamma\delta$ T cells, as well as IL-10 and TGF- β producing T cells [4, 5]. Only limited studies have been conducted on the role of regulatory T cells in autoimmune liver disease. In AIH, decreases in the frequency and function of regulatory T cells have been reported [6]. Similarly, a decrease in the frequency of regulatory T cells was observed in both patients and first-degree relatives of PBC patients [7]. Recently, murine strains exhibiting deficiencies or dysfunctions in regulatory T cells have been identified as models of PBC, displaying similar liver pathologies as well as key autoantibodies [8, 9]. This workshop presentation will summarize current research efforts involving regulatory T cells in liver autoimmunity as well as potential future directions, including eventual regulatory T cell based treatments.

- I. Introduction
 - a. Brief descriptions of PBC, AIH, and PSC
 - b. General description of regulatory T cells
- II. Autoimmune liver diseases and Tregs in humans
 - a. Primary biliary cirrhosis [7, 10]
 - b. Autoimmune hepatitis [6, 11, 12]
 - c. Primary sclerosing cholangitis [13]
- III. Regulatory T cells in animal models of liver autoimmunity
 - a. Primary biliary cirrhosis
 - i. TGF-beta receptor II dominant-negative mice [9]
 - ii. IL-2 receptor alpha(-/-) mice [8]
- IV. Future directions
 - a. Investigations into other regulatory cell subsets
 - b. Potential regulatory T cell based treatment

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Treg Therapeutic Applications and Manipulations with Regulatory T Cells

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A central goal immunologists has been to develop targeted therapies that will induce or maintain immunologic tolerance in the absence of potentially harmful immunosuppression. The ability to isolate and expand regulatory T-cell populations (Tregs) with immune suppressive activity will enable new forms of adoptive immunotherapy that may achieve this long held dream. Studies conducted during the past several decades have revealed a number of principles that will be instructive as immunotherapy with Tregs is contemplated (1,2). These principles are to 1) avoid the induction of immunogenicity of the infused cells; 2) prevent or delay cellular immunosenescence; 3) maximize help and 4) be cognizant of the known differences between mouse and human regulatory T cell biology. Most aspects of regulatory T cell biology are highly conserved between the rodent and human immune systems. However, some major differences have been uncovered that are relevant to immunotherapy. These include (i) the induction of a T regulatory type 1 (Tr1) phenotype by CD46 costimulation in humans but not mice (3); (ii) the existence of CD8⁺CD28⁻ suppressor cells in humans but not mice (4,5), and (iii) telomere degradation in human Tregs (6).

The costimulatory requirements to optimally expand natural T regulatory cells (nTREGs) for therapeutic use is unclear. We developed a cell based artificial APC system by transducing K562 cells with a variety of costimulatory molecules to explore how to optimize expansion of functional human nTREG cells (7). Regardless of the costimulatory milieu, the addition of rapamycin was necessary to consistency obtain cultures with suppressive activity. CD28 costimulation was required to maintain high levels of FoxP3 expression and enabled modest nTREG expansion in the presence of rapamycin. The addition of other costimulatory ligands such OX40-L and 4-1BBL could promote further expansion of nTREG cultures in rapamycin but these cultures often lost suppressive function, suggesting that these molecules promoted the expansion of other cell types in the presence of rapamycin. In contrast, we found that restimulation of the nTREG cell cultures after 8 days with anti-CD3 loaded CD86 aAPCs routinely resulted in 1000 fold expansion of T regulatory cells in less than 3 weeks with potent in vitro suppressive function. Lastly, we used a GVHD humanized murine model to demonstrate that purified T regulatory but not total CD4 expanded in the presence of rapamycin were able to prevent acute xenogeneic GVHD in vivo. These studies indicate the optimal costimulatory requirements to expand nTREGs differ from T effector cells and that cell based aAPCs that deliver costimulation are promising and clinically relevant approach to expand nTREGs for therapeutic use.

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Tregs in Viral Hepatitis

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CD25+ Tregs: CD4+CD25+ regulatory T cells (CD25+ Tregs) are a subset of CD4+ T cells that play a key role in self-tolerance and auto-immunity (1). While the number of Treg subsets is growing (e.g. IL10+ Tr1 cells, TGFb+ Th3 cells), CD25+ Tregs were initially defined as thymic-derived CD4 T cell subsets (natural Tregs) that highly express the IL2-receptor alpha chain (CD25) and mediate effector T cell suppression via direct cell-cell contact. Furthermore, CD25+ Treg depletion in young mice can precipitate autoimmune diseases that can be ameliorated by addition of CD25+ Tregs. With increasing knowledge about CD4+CD25+ Tregs as well as the evolving definition of their phenotypic and functional characteristics, there is increasing appreciation for their potential role in the outcome of viral hepatitis.

CD25+ Treg Markers: CD25+ Tregs display a highly activated and memory phenotype (e.g. CD45RO, HLA DR, CTLA4, GITR). Since CD25 is also upregulated in activated T cells, it is somewhat problematic to differentiate between activated and regulatory T cells. The most specific marker of CD25+ Tregs is the transcription marker FoxP3 (forkhead box protein 3) (2). The relevance of FoxP3 in immune regulation has been clearly shown by the induction of suppressive function in CD25- T-cells following forced expression of FoxP3 and by the severe immune dysregulation that occurs in-vivo in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) with FoxP3 mutation. Interestingly, FoxP3 expression can also be upregulated in previously FoxP3- cells (especially in humans), especially via direct TCR stimulation in the setting of high dose interleukin 2. Although some controversies exist, these in-vitro induced FoxP3+ cells are suppressive, raising the possibility that FoxP3+ Tregs in patients may include both natural and adaptive (induced) Tregs.

CD25+ Tregs in HCV infection: Hepatitis C virus is a highly persistent virus and chronic HCV infection occurs in the setting of functionally impaired antiviral effector T cells. CD4-mediated suppression of antiviral effector CD8 T cells was first suggested in HCV infection by the observation that CD4-depletion paradoxically augmented HCV-specific CD8 T-cell IFN γ response in some patients with chronic HCV infection (3). This study also showed that chronically HCV-infected patients display increased circulating CD4+CD25+ T cells that can directly suppress HCV-specific CD8 T-cells. Further studies showed that CD25+ Tregs can suppress CD8 T cell function in a cell-cell contact dependent manner, although there were some divergences in antigen-specificity and cytokine dependence (4-6). FoxP3 mRNA expression has been detected preferentially in CD25+ T cells in HCV-infected and uninfected persons. FoxP3 was also detected in CD8 T-cells in the context of HCV infection. Further studies in chimpanzees suggested that Tregs also contribute to immune regulation in animals that clear viremia (7). As for the liver, intrahepatic FoxP3+ cells have been recently correlated with inflammation in HCV-infected liver (8).

One conundrum about the upregulated CD25+ Tregs in HCV-infected patients is whether they represent true Tregs (rather than activated T cells). Furthermore, it is interesting to ask if these CD25+ Tregs provide preferentially HCV-specific (rather than global) immune regulation, since

global immune dysfunction (as in HIV) is generally not seen in HCV-infected patients. Our ongoing study suggests that CD4+CD25+ T cells in HCV-infected patients are bonafide Tregs that express FoxP3 and are indistinguishable from naturally occurring FoxP3+ Tregs. Moreover, we show that HCV-specific Tregs with the capacity for HCV-specific CD8 T cell suppression that can be expanded from HCV-seropositive persons, thus providing a more targeted virus-specific (rather than global) immune regulation. Further studies are needed to determine the role of FoxP3+ Tregs in liver disease progression.

CD25+ Tregs in HBV infection: Hepatitis B virus can also persist with antiviral effector T cell dysfunction, albeit mostly in vertical transmission and less frequently among immune competent adults. In one study, HBV-infected patients displayed increased circulating frequency of CD4+CD25+CTLA4+ cells. In this study, the frequency of CD4+CD25+CTLA4+ T cells in circulating lymphocytes correlated with their FoxP3 mRNA expression (9). In another study, Treg frequency was not increased in HBV-infected patients (10). However, both studies showed that CD4+CD25+ T cells from HBV-infected patients can suppress HBV-specific effector T cells in a dose-dependent manner. In another study examining a large number of HBV-infected patients, CD4+CD25+ Treg frequency was increased in peripheral blood as well as the liver of HBV-infected patients and that circulating CD4+CD25+ Treg frequency correlated with HBV titer in patients with chronic HBV infection (11). In patients with HBV-associated with hepatocellular carcinoma (HCC), circulating Treg frequency correlated with disease progression and mortality in HCC patients whereas CD4+CD25+ T cells were highly concentrated within the HCC tumor tissue (12). These results suggest that Tregs contribute to immune regulation in HBV infection and subsequent disease pathogenesis (including HCC development).

Summary and Conclusion: While the role of effector T cells in viral infection is well-described, recent studies suggest that there is a complex interplay between the effector and regulatory T cells relevant to the outcome of HBV and HCV infection. Further studies are needed to clearly define their induction and functional characteristics during HBV and HCV infection as well as their role in the initial virological outcome as well as subsequent clinical progress.

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CD8+ T Cells as Regulators of Liver Injury in Chronic HCV

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Despite considerable progress in understanding cellular immunity to hepatitis C virus (HCV), the pathogenesis of HCV-related liver injury is poorly understood. There are abundant data that cellular immune responses, especially HCV-specific CD4+ T cell responses, are related to spontaneous clearance of HCV after acute infection (1, 2). Factors including the breadth and frequency of immune response, cytokine production, and durability of response differentiate humans who clear HCV from those who progress to chronic infection. The role of immune responses in the progression of liver damage, once chronic HCV infection is established, is less clear. HCV-specific CD4+ and CD8+ T cell responses are of low frequency in chronic infection, especially in peripheral blood, making them difficult to study (3, 4). Previous cross-sectional studies are conflicting regarding whether type 1-like immune responses found in PBMC are protective from liver damage or are associated with more advanced liver disease (5), with most studies of immune correlates of liver disease severity focusing on CD4+ T cell responses. We found that in prospectively studied patients who developed acute HCV infection and progressed to chronic infection, those with strong, HCV-specific type 1 immune responses had a slower progression of fibrosis (6, 7).

There is increasing evidence that regulatory T cells (Treg) may play a critical role in the pathogenesis of HCV. Diverse populations have been shown to have regulatory activities such as CD4+CD25+Foxp3+, CD4+ cells secreting interleukin (IL)-10 or transforming growth factor (TGF)- β , as well as some CD8 T cells, natural killer T cells, and $\gamma\delta$ T cells (8). Previous studies have demonstrated populations of IL-10+CD8+ T cells, but the relationship to underlying liver injury is inconclusive (9, 10). We have found HCV specific CD8+CD25⁻FoxP3⁻ T cells from the blood of chronically infected patients suppress HCV specific T cell responses *via* TGF- β secretion (11) suggesting a role of different regulatory T cell populations in the pathogenesis of HCV infection. In subjects with chronic HCV infection, we showed that blocking regulatory-associated cytokines TGF- β but not IL-10 significantly increased HCV specific T cell responses in the peripheral blood. This was HCV-specific, since no significant effect on T cell responses to Influenza, CMV, or EBV was observed. From these observations we hypothesized that HCV-specific Treg were involved. We further demonstrated that regulatory-associated cytokine TGF- β was produced in response to HCV by a novel population of non-classic regulatory cells: CD3⁺CD8⁺CD25-negative T cells that were Foxp3 negative. Preliminary studies of these effect suggest that the suppressive effect is more pronounced in persons with slower liver disease progression. Together these observations raise the intriguing possibility that some subpopulations of CD8+ T cells may play an important role in modulating liver disease progression.

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Tregs in Liver Cancer

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Abstract

Cancers have developed powerful mechanisms to inhibit the immune system from killing them. These mechanisms include expression of checkpoint molecules such as B7H1 and B7H4. Tumor infiltration lymphocytes express high levels of PD1, the inhibitory receptor for B7H1. In addition, tumors secrete local factors that inhibit dendritic cell activation, leading to T cell tolerance as well as generation of induced regulatory T cells. Regulatory T cells play a major role in the quenching of antitumor immune responses generated by active specific immunotherapy. Pre-clinical studies, as well as a preliminary clinical trial using agents that inhibit regulatory T cells, have demonstrated increases in antitumor immune responses with this approach. Identification of both intracellular and cell membrane molecules, as well as secreted factors that mediate regulatory T cell inhibition, provides novel targets for immune based therapy of cancer.

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Young Investigator Travel Awards

These awards enable young researchers submitting abstracts for the AASLD Research Workshop to receive travel support. The 2007 award winners are:

Jong Young Choi (Abstract 584: High Circulating Regulatory T Cell Levels in Long-term Stable Liver Transplants Treated with Cyclosporine Compared to Tacrolimus). Dr. Choi will present his abstract during the Clinical Liver Transplantation and Liver Surgery Poster Session on Sunday, November 4.

High Circulating Regulatory T Cell Levels in Long-term Stable Liver Transplants treated with Cyclosporine compared to Tacrolimus

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Background/Aims: Regulatory T cell (Tregs) has been identified to play a pivotal role in the control of solid organ transplantation. In the setting of adult orthotopic liver transplantation (OLT), little is known about the extent of these cells in long-term stable transplantation recipients with maintenance immunosuppression. Methods: This prospective study enrolled total 85 (58 living donor, 27 deceased donor) consecutive recipients with sustained stable liver function over 2 years (range, 2-13 years) after OLT. We determined the frequency of CD4+CD25+ T cell in peripheral blood mononuclear cell (PBMC) of 85 liver transplantation recipients annually between January 2005 and December 2006. In 10 liver biopsy tissue during study period, Foxp3+ cells were analyzed using immunohistochemistry. We investigated the possible link between immunosuppressive drug and the frequency of CD4+CD25+ T cell. Results: In overall patients, the mean frequency of Tregs was 4.7% (range, 1~17.3). When the cut-off level of high frequency of CD4+CD25+ Treg cell was arbitrarily defined over 6% among CD4+ T cells, 29.4% (25/85) of long-term stable recipients showed high frequency of Tregs cell over 2 years after OLT. The proportion of patients with high Tregs over 6% was 41.1% (12/29) in CsA group, 13.8% (4/29) in FK506 group, especially in living donor liver transplantation (LDLT) (P=0.03). Eight of 10 patients had Foxp3 positive cells in liver biopsy tissue. Conclusions: If 6% Tregs among PBMC was arbitrarily the cut off level of Tregs cell frequency predicting clinical tolerance, approximately one fourth of 85 long-term stable liver transplantation recipients meet this criteria. In addition, this study suggests that cyclosporin A may induce the tolerance than FK506 in long-term stable LDLT patients.

Mark Claassen (Abstract 1035: Intrahepatic Regulatory T Cells Differ Phenotypically from their Circulating Counterparts in Chronic Therapy Naïve HCV Patients). Dr. Claassen will present his abstract during the Inflammation and Immunobiology Poster Session on Monday, November 5.

Intrahepatic Regulatory T cells differ phenotypically from their circulating counterparts in chronic therapy naïve HCV patients

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BACKGROUND: Regulatory T cells (Treg) play a significant role in the hampered immune response against HBV, as we have shown previously. Similar findings have been suggested for Treg in chronic HCV infection. This may explain the weak HCV specific T cell response in these patients. The immune response at the primary site of infection, the liver, is likely to play a critical role in HCV persistence. **METHOD:** Minimally invasive aspiration biopsies enabled us to repeatedly study intrahepatic and peripheral Treg in parallel.

RESULTS: In 20 chronic HCV therapy naïve patients, CD8+ lymphocytes were more frequent in the liver than in blood (22.8% and 13.6% respectively, $p < 0.001$). In contrast, the proportion of CD4+ lymphocytes was lower intrahepatically than peripherally (26.34% and 31.57% respectively, $p < 0.05$). Careful analysis of CD4+CD25^{hi} cells and FoxP3+CD4+CD25+ cells revealed a smaller proportion of Treg in the liver (8.1% against 9.6%). Preliminary data suggest that Treg in liver and in blood predominantly consist of a memory phenotype (a.o. CD45RO). Our findings indicate that a significant proportion of CD4+ lymphocytes infiltrating the liver are Treg. However, the ratio Treg to total CD4+ lymphocytes and total CD8+ lymphocytes was lower than in peripheral blood.

CONCLUSIONS: Our data indicate that the chronicity of HCV can not be solely explained by an increased ratio of Treg to effector T cells in the liver. We are currently further investigating phenotypical and functional differences between liver and blood Treg. This could delineate the contribution and possible mechanism of intrahepatic Treg to the attenuated HCV immunity.

Malte H. Heeg (Abstract 146: Chronic HBV Evolution is Associated with Numeric and Phenotypic Changes in Peripheral HBV-core Epitope Specific CD4+ T-cells: A Study Using a Novel HBV-core Specific HLA-DRB1*0101 Tetramer for the Analysis of Antiviral CD4+ T-Cell Responses During Acute and Chronic Hepatitis B). Dr. Heeg will present his abstract during the Innate and Adaptive Immunity in Liver Diseases Parallel Session on Monday, November 5.

Chronic HBV evolution is associated with numeric and phenotypic changes in peripheral HBV-core epitope specific CD4+ T-cells: A study using a novel HBV-core specific HLA-DRB1*0101 tetramer for the analysis of antiviral CD4+ T-cell responses during acute and chronic hepatitis B

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A strong HBV-specific CD4+ T cell response is associated with spontaneous viral clearance in acute hepatitis B but is weak or short-lived in patients developing chronic disease. So far, this CD4+ T-cell response could just be investigated by functional assays which lack the possibility of further T-cell phenotyping and that are unable to identify dysfunctional cells. HBV-specific HLA-class-II tetramers now can negotiate those limitations.

To establish a new HBV-core MHC-class-II tetramer we screened 33 patients with acute HBV infection by overlapping HBV-core peptides and identified a HLA-DRB1*0101 restricted HBV-core epitope. This epitope was recognized by all DRB1*0101 patients with acute HBV infection as well as by 77 % (17/22) of all patients with acute HBV. Using this epitope we assembled a new DRB1*0101 MHC-class-II tetramer. The tetramer specifically stained a corresponding CD4+ T cell clone. To proof the tetramer sensitivity dilution experiments of Tet+ clone cells into PBMC were performed and a strong correlation between added and measured frequencies was found. In six HBV-negative DR1+ individuals and nine patients with acute hepatitis B lacking DR1, no CD4+ cells were tetramer positive. In three HLA-DR1+ patients with acute hepatitis B, HBV-specific CD4+ T cells were detectable during the acute phase of disease, ranging from 900 to 1680/106 CD4+ T cells during the peak phase. The peak phase was associated with an ALT peak and viral clearance as well as with a peak level of IFN- γ secretion. The frequencies of HBV specific CD4+ T-cells decreased rapidly after viral clearance but were detectable in considerable levels over several months. In contrast, at different time points of disease 15 investigated patients with chronic hepatitis B showed no or only very low frequencies of tetramer positive cells. During the course of acute self limiting hepatitis B, phenotypic characterization demonstrated a rapid increase of IL7R and CD62L expression on Tet+/CD4+ T-cells.

In conclusion, using a new MHC-class-II tetramer containing one immunodominant HLA-DRB1*0101 restricted epitope within HBV-core, we were able to quantify and characterize HBV specific CD4+ T cell responses patients with acute hepatitis B and chronic hepatitis B. In patients with chronic disease almost no Tet+/CD4+ T-cells were found indicating a complete loss of antiviral CD4+ T-cells rather than a loss of function of those cells. Furthermore those cells showed an impaired capacity to develop a memory phenotype. So far tools which allow to distinguish between the absence of specific cells or their non-function were not available.

Ichiyo Itose (Abstract 473: Involvement of Regulatory T Cell Dynamics in the Achievement of Biochemical Response in 48-week PEG-IFN 2b and Ribavirin Combination Therapy for Chronic Hepatitis C Patients). Dr. Itose will present his abstract during the Viral Hepatitis: Pathobiology Poster Session on Saturday, November 3.

Involvement of regulatory T cell dynamics in the achievement of biochemical response in 48-week PEG-IFN α 2b and ribavirin combination therapy for chronic hepatitis C patients

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Aim: In Peg-IFN α and ribavirin combination therapy for chronic hepatitis C, the therapy might be discontinued in patients who fail to become negative HCVRNA by week 24 of the therapy, since the probability of SVR in them is extremely low. It is reported that the progression of fibrosis is slow and the incidence of hepatocellular carcinoma is low in patients who achieved biochemical response (BR), even if they failed to eradicate HCV. Therefore, in order to prevent hepato-carcinogenesis in patients who fail to eradicate HCV, it is clinically important to continue the therapy in patients who would achieve BR.

Regulatory T cells (Treg) are capable of suppressing antigen-specific and non-specific T cell responses, which is reported to be increased in chronic hepatitis C. We reported that Treg function is enhanced in patients with persistently normal ALT levels (PNALT) than in patients with active hepatitis, suggesting that Treg is involved in alleviating liver inflammation. We aimed to clarify whether the changes of blood cell subsets could be served as marker for defining BR patients in PEG-IFN α and ribavirin therapy.

Method: Thirty patients who received PEG-IFN α 2b and ribavirin combination therapy for 48 weeks were enrolled in the study. Before treatment and at weeks 8, 12, 24, and 48 during the therapy, PBMCs were collected from the patients. The frequencies of DC subsets (MDC and PDC), Th1, Th2 cells and Treg (CD4+CD25high+) and their changes from the beginning of the therapy were determined by flowcytometric analyses.

Result: Among the patients who completed the treatment, 12 patients achieved SVR, 12 were TR and 6 were NR, respectively. The SVR patients showed significant smaller pre-treatment frequency of MDC when compared to TR patients ($p < 0.01$). In contrast, PDC frequency did not differ among the groups. The frequency and its changes of Treg during therapy were not different among SVR, TR and NR groups, suggesting that Tregs possess minor roles in virological response. 22 patients attained BR and the remaining 8 were non-BR at the end of treatment. The frequency of Treg in BR group significantly increased after the beginning of the therapy ($p < 0.05$), whereas it did not in non-BR patients. Even in patients with positive HCVRNA at the 24th week of the therapy, Treg in BR showed significant increase after starting of the therapy ($p < 0.05$). In parallel with such increase of Treg, plasma TGF- β 1 level in BR patients was higher than those in non-BR, suggesting its possible role in Treg induction.

Conclusion: Increase of Tregs may be involved in biochemical response in PEG-IFN α and ribavirin combination therapy, being independent of virological response.

Gadi Lalazar (Abstract 149: A Combination of Beta-glycolipids Overcomes a CD1d Dependent Inhibition of NKT Lymphocytes: An Alternative Flotillin-2 Raft Protein Dependent Regulatory Cell Activation Pathway). Dr. Lalazar will present his abstract during the Innate and Adaptive Immunity in Liver Diseases Parallel Session on Monday, November 5.

A Combination of beta-glycolipids overcomes a CD1d dependent inhibition of NKT lymphocytes: An alternative Flotillin-2 raft protein dependent regulatory cell activation pathway

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CD1-restricted natural killer T (NKT) cells are regulatory lymphocytes that co-express a conserved T-cell and natural killer (NK) receptors. Lipid rafts are highly dynamic, submicroscopic assemblies enriched in sphingolipids and cholesterol. CD1d, a raft-localized receptor, is an essential restriction element in raft glycolipid-mediated activation. Lipid raft disruption inhibits NKT cell activation but does not interrupt ligand loading onto CD1d, in contrast to direct blocking of the CD1d receptor by a monoclonal antibody. Raft disrupters were shown to inhibit IL-6/STAT3 and IFN- γ /STAT1 signaling suggesting that almost all signaling by these cytokines is initiated in raft microdomains. Purpose: To determine the signaling pathway of NKT activation using naturally occurring beta glycolipids, and its dependence on the CD1d membrane receptor. Methods: NKT hybridoma cells (DN32.D3) were stimulated in vitro using 500ng/ml of beta-glucosylceramide (GC), beta-lactosylceramide (LC), a 1:1 combination of both (IGL), or PBS. The detergent-insoluble membrane complexes were floated on nycodenz gradient and their GM1 content was analyzed by dot blot. NKT signaling pathway was assessed using western-blot analysis of Flotillin-2, LCK STAT1 and 3. NKT activation was measured using a 3[H] thymidine incorporation assay. To determine the effect of CD1d on the signaling pathway, anti-CD1d monoclonal blocking antibodies were used. Results: Administration of IGL led to a more prominent flotillin-2 recruitment to the raft fractions (1-2) compared with GC, LC and PBS. No effect was noted on LCK and GM1 in all treatment groups. All glycolipids led to a significant increase in STAT1 expression in the cytosolic fractions (8-12), but STAT3 expression was prominent for IGL only. Administration of IGL, resulted in stimulation of NKT cell proliferation, in contrast with an inhibition with GC or LC alone (increase by 18%, versus a decrease by 3% and 10%, respectively, $p < 0.05$). The addition of anti CD1d antibodies resulted in increased cell proliferation for GC, LC, IGL compared with PBS (13%, 27%, and 12% respectively, $p < 0.05$). Conclusion: Administration of the combination of beta-glycolipids, IGL overcomes the CD1d dependent inhibition of NKT activation by naturally occurring beta glycolipids. An alternative membrane raft Flotillin-2 mediated pathway may be an important mechanism for the effect of these novel compounds on regulatory cells. These data suggest that IGL may serve as a novel immune modulator in NKT mediated liver disease.

Maria Serena Longhi (Abstract 722: Effect of CD4+CD25+Regulatory T-cell on Monocyte Toll-like-Receptor-4 Expression in Autoimmune Hepatitis). Dr. Longhi will present her abstract during the Human Cholestatic and Autoimmune Liver Disease Poster Session on Sunday, November 4.

Effect of CD4+CD25+ regulatory T-cell on monocyte Toll-like-receptor-4 expression in autoimmune hepatitis

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Background: CD4+CD25+ T-regs control adaptive immunity preventing autoimmunity. Little is known on T-reg effect on innate immunity. Monocytes, cells of the innate immune system, after stimulation by their natural ligand lipopolysaccharide (LPS) through Toll-like-receptor-4 (TLR4), trigger adaptive immune responses. Monocytes abound in the portal/periportal cellular infiltrate in autoimmune hepatitis (AIH).

Aim: To investigate the effect of T-regs on monocyte TLR4 expression in the presence or absence of LPS in AIH.

Patients and methods: 16 patients with ANA/SMA+ve AIH (median age: 14.7 years; 7 females) and 7 normal controls (median age 28 years; 7 females) were studied.

Monocytes, T-regs and CD4+CD25- T-cells were isolated from peripheral-blood-mononuclear-cells using immunomagnetic beads. Monocyte TLR4 expression (assessed as mean fluorescence intensity) was evaluated by flow cytometry before and after 24-hour co-culture with T-regs (or with CD4+CD25- T-cells as control) in the absence or presence of LPS. To assess whether T-reg effect on monocytes is due to cell-to-cell contact and whether is mediated by IL-10, transwell experiments and neutralization assays using anti-IL-10 antibodies were performed.

Results: Before LPS stimulation and in the absence of T-regs the expression of TLR4 on monocytes was similar in controls and patients. Following T-reg addition it increased by 29% (from 34.6±4.9 to 44.5±5.8, P=0.03) in controls and by 39.8% (from 41.9±3.4 to 58.7±6.7, P=0.025) in AIH. Following LPS stimulation in the absence of T-regs, monocyte TLR4 expression increased by 21% (P=0.1) in normal and by 15.9% (P=0.1) in AIH. After T-reg addition in the presence of LPS, TLR4 increased by 23% (from 42.5±4.2 to 52.3±7.7; P=0.046) in controls and by 33% (from 48.7±3.5 to 64.6±5.1; P=0.016) in AIH. Following T-reg addition, the expression of TLR4 on monocytes was higher in patients than in controls both in the absence (P=0.12) and presence (P=0.059) of LPS. No effect on monocyte TLR4 expression was observed when CD4+CD25- T-cells were added instead of T-regs. T-reg effect on monocyte was mainly mediated by a cell-to-cell mechanism, the increased TLR4 expression being lower (17% in normal and 16% in AIH) when monocytes were kept separated from T-regs than when they were in direct contact (29% and 39.8% respectively). Increased TLR4 expression was inhibited by addition of anti-IL-10 by 86% in controls and 91% in patients.

Conclusions: Unexpectedly, T-regs increase TLR4 on monocytes through cell-to cell contact in the presence of IL-10, especially in AIH. The significance of this phenomenon in the pathogenesis of AIH remains to be clarified.

Masashi Sasaki (Abstract 1043: Intrahepatic Status of Regulatory T Cells in Autoimmune Hepatitis, PBC, Chronic Hepatitis C, and Chronic Hepatitis B). Dr. Sasaki will present his abstract during the Inflammation and Immunobiology Poster Session on Monday, November 5.

Intrahepatic status of regulatory T cells in autoimmune hepatitis, primary biliary cirrhosis, chronic hepatitis C, and chronic hepatitis B

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Background/Aims: Regulatory T cells (Tregs) maintain immunological tolerance and suppress autoreactive immune responses. We evaluated the intrahepatic status of Tregs in patients with autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), or chronic hepatitis B (CH-B).

Methods: We analyzed 85 patients (20 AIH, 22 PBC, 27 CH-C, and 16 CH-B) and 14 controls. Using liver tissue samples obtained by needle biopsy or from marginal parts of resected metastatic liver tumors in controls, immunohistochemical analyses of forkhead box P3+, which is a specific marker for Tregs, CD4+, and CD8+ cells were performed.

Results: Intrahepatic Tregs were significantly infiltrated in patients with liver diseases than in controls. There were significantly fewer intrahepatic Tregs in AIH patients than in PBC patients (p=0.037). Patients with a low frequency of intrahepatic Tregs were detected significantly less in the AIH and CH-B groups than in the PBC and CH-C groups. We found significantly less infiltration of CD4+ T cells in AIH than in other diseases (p<0.05). Liver-infiltrating CD8+ T cells were detected more frequently in the CH-B group than in other groups (p<0.003).

Conclusions: Intrahepatic Tregs were increased in both patients with autoimmune liver diseases and those with viral hepatitis. In autoimmune liver diseases, intrahepatic Tregs in AIH patients were fewer than in PBC patients.

Frequency of intrahepatic FOXP3+, CD4+, and CD8+ cells in patients with each liver disease.

	AIH	PBC	CH-C	CH-B	Control
%FOXP3+	5.2±2.4*,**	7.2±3.5*,**	6.2±3.7**	6.3±1.6**	1.1±1.1**
%CD4+	37.8±11.0†	47.4±11.0 †	44.0±9.3 †, ¶	50.6±8.7 †, ¶, Ω	41.0±10.9Ω
%CD8+	32.2±12.2‡	33.3±10.4 ‡	30.5±7.0 ‡	44.2±9.3 ‡	32.0±1.9‡

The values are means ± SD. *p = 0.037 (AIH vs PBC); **p < 0.001 (Patients vs Control) †p = 0.007, 0.045, 0.001 (AIH vs PBC, CH-C, CH-B); ¶p = 0.026 (CH-C vs CH-B); Ω p = 0.013 (CH-B vs Control); ‡p = 0.003, 0.002, 0.001, 0.001 (CH-B vs AIH, PBC, CH-C, Control).

