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Molecular Linking of HIPEC (Hyperthermic Intraperitoneal Chemotherapy) and Tregs (Regulatory T- cells) in Advanced Epithelial Ovarian Cancer - A Review

RAY MD[1]*, Deo SVS[2], Luthra K[3], Mathur S[4], Anand P[5] and Sharma A[6]

[1]*Additional Professor, Department of Surgical Oncology, IRCH, AIIMS, New Delhi, India*
[2]*Professor, Department of Surgical Oncology, IRCH, AIIMS, New Delhi, India*
[3]*Professor, Department of Biochemistry, AIIMS, New Delhi, India*
[4]*Professor, Department of Pathology, AIIMS, New Delhi, India*
[5]*Senior Resident, Department of Surgical Oncology, IRCH, AIIMS, New Delhi, India*
[6]*Assistant Professor, Department of Biochemistry, AIIMS, New Delhi, India*

Abstract

The mainstay in the management of advanced epithelial ovarian cancer is platinum-based chemotherapy and complete cytoreductive surgery. Despite this, about two-thirds of patients have disease recurrence mostly within the peritoneal cavity. HIPEC (Hyperthermic Intraperitoneal Chemotherapy) is a modality that delivers cell-cycle non-specific chemotherapeutic agents along with heat 41°C to 43°C into the peritoneal cavity. HIPEC is done intra-operatively after achieving complete cytoreduction (which means after removal of all the tumor deposits more than 2.5 mm). Ovarian cancer is associated with the frequent finding of tumor-infiltrating lymphocytes in their tissue microenvironment. Especially studies have shown that ovarian cancer evades immune surveillance by higher expression of *FOXP3* T cells. HIPEC has been used in the treatment of primary and recurrent tumors. In this review, we discuss how the significance of HIPEC on genetics and immunology of these patients with cancer have provided unique insights into the molecular and cellular basis of Treg cells. Studies of HIPEC and its association with Tregs cells should make it possible to increase the paucity of immuno- therapeutic modalities of most human cancer at an unprecedented level of molecular and cellular precision. The predictive, preventive, and therapeutic implications of these studies of HIPEC in relation to immunity in EOC may extend to patients with other peritoneal carcinomatosis.

Keywords: Ovarian cancer • HIPEC • Regulatory T-Cells • Immunotherapy • Peritoneal carcinomatosis

Abbreviations: HIPEC: Hyperthermic Intraperitoneal Chemotherapy • *FOXP3*: Forkhead Box P3 • EOC: Epithelial Ovarian Cancer • Treg: Regulatory T-Cells • CRS: Cytoreductive Surgery • NACT: Neo-Adjuvant Chemotherapy • HSP: Heat Shock Proteins • nTregs: natural Treg cells • iTregs: Inducible Treg Cells • CD: Cluster of Differentiation • CTLA-4: Cytotoxic T-Lymphocyte Antigen 4 • LAG-3: Lymphocyte-Activation Gene 3 • 4-IBB: Belongs to the Tumor Necrosis Factor Receptor Superfamily • IL: Interleukin • NK Cells: Natural Killer Cells • TAA: Tumor-Associated Antigens • APC: Antigen Presenting Cells • TGF: β Transforming Growth Factor • MHC: Major Histocompatibility Complex • RORγ : Retinoic Acid Receptor-Related Orphan Nuclear Receptor Gamma • Th Cells: T-Helper Cells • LTi cells: Lymphoid Tissue Inducer Cells • LBD: Ligand-Binding Domains • ELISA: Enzyme-Linked Immune Sorbent Assay • PB: Peripheral Blood • PF: Peritoneal Fluid • TNF: Tumor Necrosis Factor • IFN: Interferon • TLR: Toll-Like Receptor • HDAC: Histone De-Acetylase • IRCH: Institute Rotary Cancer Hospital • AIIMS: All India Institute of Medical Sciences • ECOG: European Oncology Co-operative Group • RNA: Ribonucleic Acid • cDNA: Complementary DNA • DNA: De-oxy Ribose Nucleic Acid • dNTP: Deoxynucleoside Triphosphates • RT: Reverse Transcriptase • PCR: Polymerase Chain Reaction • BSA: Bovine Serum Albumin • IHC: Immunohistochemistry • ANOVA: Analysis of Variance

Introduction

In advanced Epithelial ovarian cancer (EOC), a subgroup of patients has disease recurrence despite having a good clinical response to platinum-based chemotherapy and complete cytoreductive surgery. A growing number of immune cells have been deciphered over several years. The frequent finding of tumor-associated lymphocytes, as well as differential immunological markers (tumor-associated antigen) from the tumor specimen, provides explicit evidence of immunological response to ovarian cancer. This immune response may have implications with regard to disease prognosis.

In the context of several treatment modalities, $^{2}/_{3}^{rd}$ of these patients will eventually relapse. The peritoneal cavity is the most common site of relapse. Intraperitoneal therapies have been advocated to command the disease progression. In this context, HIPEC (Hyperthermic intraperitoneal chemotherapy) is one of the effective IP therapies which boosts the therapeutic efficacy by killing tumor cells and inducing an efficient anticancer immune response.

The nomenclature of HIPEC depends on the timing of the intervention in relation to systemic chemotherapy and was described by Mulier et al. It is called as Upfront/ primary CRS and HIPEC -when performed primarily, Interval CRS and HIPEC- when performed after 6 six cycles of NACT, consolidation HIPEC- when done in patients with a complete clinicoradiological response after NACT, secondary CRS and HIPEC-when performed for patients who have a recurrence after CRS or CRS+HIPEC and Salvage CRS + HIPEC-in

***Address for Correspondence:** Mukurdipi Ray, Additional Professor, Department of Surgical Oncology, IRCH, AIIMS, New Delhi-110049, India, E-mail: drmajormdrayaiimsdelhi@aiims.edu*

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re-recurrent cases after secondary CRS + HIPEC. However, the fine detail of the molecular mechanisms of HIPEC underlying the association with the immune response is unknown. Here we are endeavoring immense knowledge of immunological aspects of HIPEC in epithelial ovarian cancer by including some evidential fact that HIPEC may be associated with Treg expression.

In this review, we discuss how the significance of HIPEC on genetics and immunology of these patients with cancer, has provided unique insights into the molecular and cellular basis of Treg cells. Studies of HIPEC and its association with Tregs cells should make it possible to increase the paucity of immuno-therapeutic modalities of most human cancer at an unprecedented level of molecular and cellular precision. The predictive, preventive, and therapeutic implications of these studies of HIPEC in relation to immunity in EOC may extend to patients with other peritoneal carcinomatoses.

Literature Review

Molecular interaction between HIPEC and Treg

Role of hyperthermia: ....In HIPEC, heated chemotherapeutic agents are perfused into the peritoneal cavity at 41-43°C with a dwelling time of 45 to 90 min. Although perfusion is increased by hyperthermia in normal tissue, opposite observations were made in tumor tissues with reduced perfusion leading to hypoxia in the tumor cells [1]. Hyperthermia induces the expression of heat shock protein (HSP), thus able to regulate a series of cellular and molecular effects evoking the complex immunological alterations in the host microenvironment. In unstressed cells, HSPs are chaperone proteins that maintain protein configuration and transport. They act as a defense mechanism toward apoptosis-inducing events. The overexpression of HSPs in various tumor entities is associated with poor prognosis. Studies have shown that cytostatic treatment for 1 hour using 5-Fluorouracil, Mitomycin-C, or Oxaliplatin under hyperthermia resulted in significantly increased gene expression of HSPs (especially HSP27, HSP70, and HSP90) compared with normothermic conditions (37°C) as well as corresponding hyperthermic conditions alone without chemotherapy. Even 3 days after exposure, increased HSP expression profiles were observed suggesting similar effects *in vivo* after a HIPEC procedure [1].

HSPs interact with several survival and anti-apoptotic pathways allowing the cell to deal with potentially lethal conditions. The heat shock proteins provide a defense mechanism by linking up with antigen presentation, cross-presentation, and anti-tumor immunity which all interact with the Regulatory T cells (Tregs) expression (Figure 1). From various studies, it has been proved that Tregs remarkably enhance the immune-suppressive mechanism in the tumor microenvironment of ovarian cancers.

Based on their interference with apoptotic pathways, HSPs have become an interesting target for inhibitory strategies in cancer therapy. Many potential

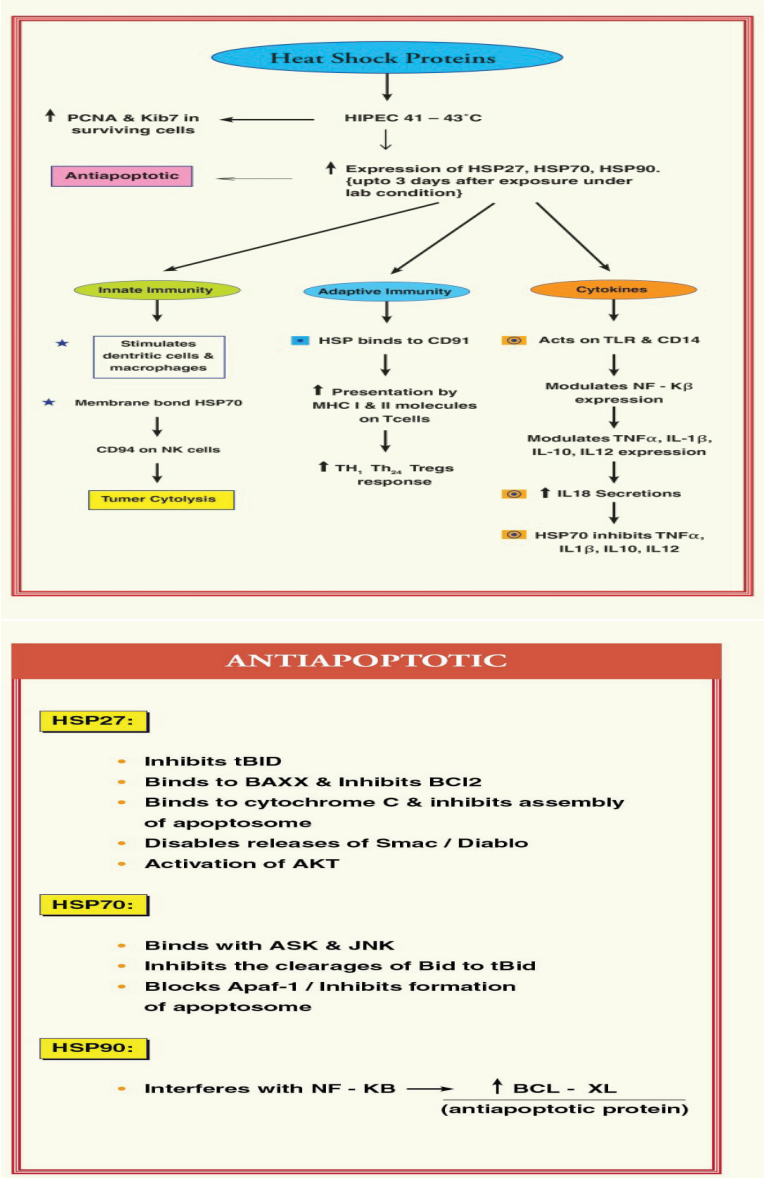

Figure 1. Complex molecular interaction of heat shock proteins.

inhibitors of particularly HSP90 have been tested for cancer therapy and currently, HSP90 inhibitors are being evaluated in numerous clinical trials [2]. Next to Ganetespib (STA-9090), Luminespib (AUY922) in combination with 5-FU and OXA has been described to show a synergistic antiproliferative effect in gastric cancer [2,3]. As HSP90 inhibition has been demonstrated to induce the expression of HSP70, dual targeting of HSP70 and HSP90 might be beneficial to successfully inhibit HSP-dependent antiapoptotic mechanisms [4]. Different small-molecule HSP70 inhibitors such as VER155008 and 2-phenylethynesulfonamide have already been proven to enhance the cytotoxicity of HSP90 inhibitors in oral squamous cell carcinoma [5-7]. For targeting HSP27, 2 small-molecule inhibitors, quercetin and brivudine (RP101) exhibits anti-tumor effects in the prostate, breast, squamous cell, ascites, and gastric cancer cell lines. Similar to AUY922, quercetin potentiates the effects of many first-line chemotherapeutics including doxorubicin, cisplatin, gemcitabine, and 5-FU [8-10]. Interestingly, studies on cell viability data from HSP inhibition assays, using a combination of HSP90 (17-AAG) and HSP70 (VER155008) inhibitors, indeed demonstrated significantly reduced viability of colon cancer cells previously exposed to hyperthermic chemotherapy, suggesting a beneficial effect of HSP inhibition after HIPEC therapy.

Regulatory T-cells: Treg cells can be described as a T-cell population that functionally suppresses an immune response by influencing the activity of another cell type [11,12]. Treg cells were initially described by Gershon et al. in the early 1970s and were called suppressor T cells [13]. Two main groups of Tregs have been characterized based on their site of development i.e., in the thymus (natural Tregs –nTregs) and the periphery (inducible Tregs-iTregs) [14]. NTregs are derived from bone marrow progenitor cells transported to the thymus where they differentiate into nTregs following negative and positive selection. Following maturation, these cells migrate to the periphery [15]. NTregs can be differentiated from other T cells owing to the exclusive expression of forkhead box P3 (*FOXP3*) which is necessary for nTreg-suppressive function [16,17]. Other essential effector and costimulatory molecules that are expressed by these cells include CD39, CD73, cytotoxic T-lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), CD28, CD80/86, CD40, OX40 (CD134), and 4-IBB (CD137) [18-20].

iTregs are generated from naive $CD4^+$ T cells subsequent to induction by IL-10 and TGF-β resulting in two populations of iTregs, type 1 Tregs (Tr1), and T helper 3 (Th3) cells, respectively [21-23]. The suppressive function of these cells occurs via IL-10 and TGF-β. Peripheral Tregs can also be generated through interactions between IL-4 or IL-13 and the IL-4Rα. Although *FOXP3* is a characteristic marker of nTregs, Th3 cells can also be induced to generate *FOXP3* [24-26].

Upon activation of the T cell receptor, Tregs suppress dendritic cells, B cells, macrophages, osteoblasts, mast cells, NK cells, NKT cells, $CD4^+$ T, and $CD8^+$ T cells [27]. This is an important "self-check" built into the immune system to prevent excessive reactions i.e., peripheral tolerance. Since tumor-associated antigens (TAA) are autoantigens, they are subjected to control by peripheral tolerance.

Mechanism of immune modulation by Treg: Tregs suppressive mechanisms transpire through the following mechanisms viz., cytokine secretion, cytolysis, metabolic destruction, and altering of APCs function. The secretion of inhibitory cytokines such as IL-10, TGF-β, and IL-35 suppresses immune function. The inhibitory effects of IL-10 occur via its association with APCs to suppress inflammation hence, in the absence of IL-10-secreting Tregs there is an increase in inflammation [28]. IL-35 on the other hand suppresses the expansion of T cells into other T helper subsets, as well as B cells and macrophages [29,30]. TGF-β is necessary for the survival of Treg subsets [31] (Figure 2). Tregs may suppress the function of other cells via granzyme-mediated killing following the release of granzymes into the target cells [32,33]. Similarly, metabolic disruption involving induction of adenosine and the production of cyclic adenosine monophosphate (cAMP) may be a vital mechanism for suppressing overreactive cells [34]. The versatility in Treg effector function allows them to modulate innate immune cells in particular APCs. This entails the engagement of surface molecules such as CTLA-4 and LAG-3 with MHCII molecules on the APCs conferring inhibitory responses that avert the stimulation of other conventional T cells [35]

***FOXP3* gene**: *FOXP3* (forkhead box P3), also known as scurfin, is a protein involved in immune responses [36]. The gene of *FOXP3* is located on the short arm of the X chromosome at Xp.11.233. *FOXP3* is a transcription factor that is necessary for the induction of the immunosuppressive functions of regulatory T cells (Treg) (Figure 3). Tregs within the CD4+ CD25+ T cell population are characterized by the expression of the *FOXP3*+ protein [37,38].

Humans bearing tumors show an elevated amount of Tregs in their blood as well as malignant effusions [39,40]. *FOXP3* is reported to be expressed in various kinds of tumor cells including colorectal cancer, melanoma, non-small cell lung cancer, and many other cancer cell lines [41-43]. Sato et al. identified cells in ovarian tumors expressing both CD25 and *FOXP3*. Recently, the presence of Tregs in ovarian cancer ascites in comparison to normal ascites has been shown [40].The presence of Tregs in ovarian tumors has been associated with reduced overall survival [44,45]. More specifically, Curiel et al. showed for the first time that CD4+ CD25+ *FOXP3*+ Treg cells correspond to poor clinical outcomes in epithelial ovarian cancer [44]. The same study also showed that CD4+ CD25+ CD3+ cell populations were much more

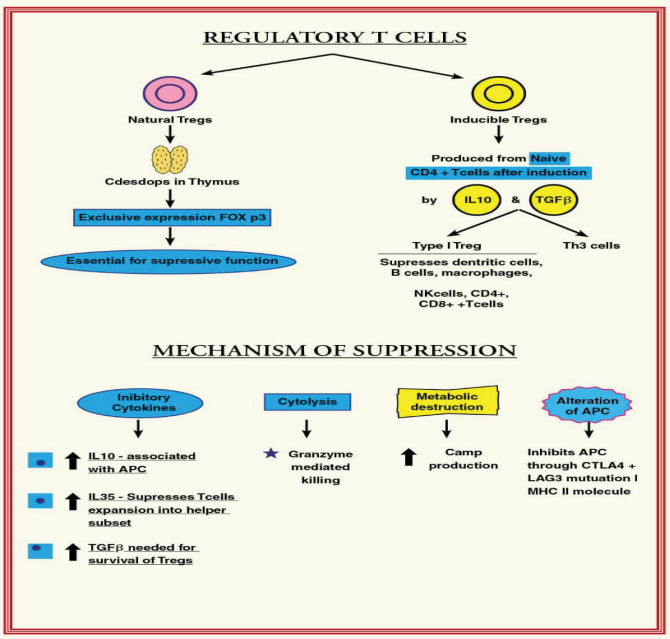

Figure 2. Mechanism of immune modulation by Treg.

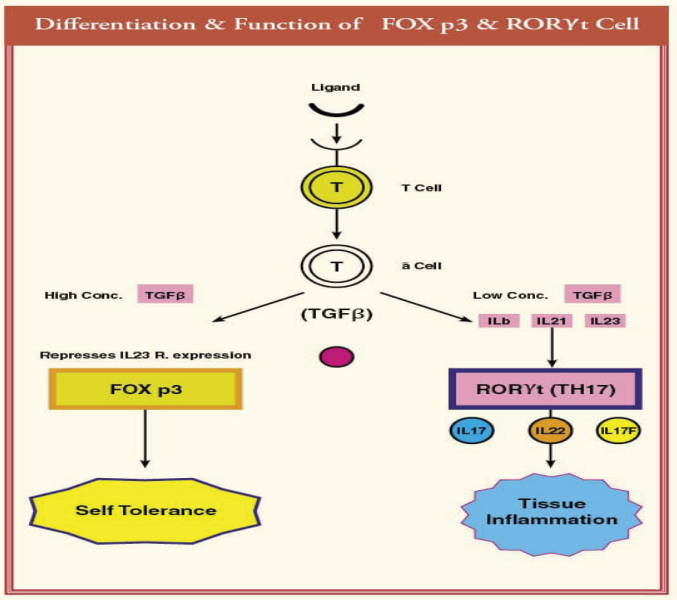

Figure 3. Differentiation and function of FOXp3 and RORγT cells.

concentrated in malignant ascites rather than nonmalignant ones and blood. It was also shown that CD4+ CD25+ cells were preferentially concentrated in tumor mass rather than in tumor-draining lymph nodes. Furthermore, the presence of *FOXP3* alone was an independent prognostic factor for progress-free and overall survival.

RORγ Th17 Cells: Nuclear hormone receptors (NHRs) form a family of transcription factors that are composed of modular protein structures with DNA- and ligand-binding domains (DBDs and LBDs). The DBDs confer gene target site specificity, whereas LBDs serve as control switches for NHR function.Th17 cells are CD4+ T helper effector cells that express several pro-inflammatory cytokines including interleukin-17A (IL-17). The NHR RORγt, an immune cell-specific isoform of RORγ (retinoic acid receptor-related orphan nuclear receptor gamma) is a key transcription factor for the development of Th17 cells.

RAR-related orphan nuclear receptor gamma (RORγ) has seen a renewed interest as a potential treatment for a variety of disorders like rheumatoid arthritis, autoimmune disorders, multiple sclerosis, psoriasis, psoriatic arthritis (Figure 3). The pathology of the multiple human autoimmune diseases has shown the involvement of T helper 17 cells and inflammatory cytokines such as IL-17A and IL-17F. Antibodies against IL-17 and IL-23 (Risankizumab) have validated the IL-23 and IL-17 pathway in psoriasis. RORγ exists in two isoforms.

RORγ (also referred to as RORγ1) is expressed in the lung, liver, kidney, muscle, brown fat, and thymus. Furthermore, even though RORγ1 is abundantly expressed, detecting the protein has been challenging. However, RORγt is predominantly highly expressed in the thymus, where it has been identified in immature $CD4^+/CD8^+$ thymocytes as well as lymphoid tissue inducer (LTi) cells. The ligand-binding domains (LBDs) of RORγ1 and RORγt are identical, and the crystal structures of the LBD of RORγt with various ligands have been reported.

In a study conducted by Monika Bilska et al. they investigated the potential role of Th17 cells in ovarian cancer patients (n = 71) by analyzing the frequencies of Th17 cells in three different environments, i.e., peripheral blood (PB), peritoneal fluid (PF), and tissue (Th17 infiltrating cells), and the concentration of IL-17A in plasma and PF of patients in terms of their clinical and prognostic significance [46.] Th17 cells were analyzed by flow cytometry as a percentage of CD4+ lymphocytes that expressed intracellular expression of IL-17A. The level of IL-17A in plasma and PF were determined by ELISA. Results showed the accumulation of Th17 cells among tumor-infiltrating CD4+ lymphocytes (in relation to PB). Moreover, the percentage of Th17 cells in both PB and PF of OC patients was significantly lower than that in the benign tumors group (n = 35). There were no significant differences in the percentage of Th17 cells in PB, PF, and tissue in relation to clinicopathological characteristics of OC patients and survival. The lower percentage of Th17 cells in the PB and PF of OC patients may promote evasion of host immune response by cancer cells. The concentration of IL-17A in plasma of OC patients was higher than that in both benign tumors and the control group (n = 10). The PF IL-17A level in OC patients was higher than that in women with benign ovarian tumors, indicating its synthesis in the OC microenvironment. Higher IL-17A level in PF is correlated with longer (median: 36.5 vs. 27 months) survival of OC patients.

Interaction between HSP and Treg cells: HSPs are implicated in both the adaptive and innate immune systems. In the innate immune system, HSPs stimulate dendritic cells and macrophages, as these are APCs, they consecutively stimulate adaptive immune cells [47,48]. HSPs are important in NK cell function as they are known to increase cytotoxic function and cell proliferation [49]. In particular, membrane-bound HSP70 on various cancer cells is recognized by cluster of differentiation (CD)-94 on the NK cell, initiating effective cytolysis of the tumor cells [50,51]. HSPs may induce the secretion of either anti- or proinflammatory cytokines thereby monitoring the immune response [52,53].

Similarly, HSPs increase the effectiveness of cross-presentation between antigens and APCs in the extracellular milieu, perpetrating in the presentation of peptides to major histocompatibility complex class one (MHCI) or MHCII molecules on T cells. CD91, an HSP receptor, is a requisite for this process and increases T-cell-mediated responses related to T helper (Th)1, Th2, and Tregs the presence of tumors, concomitant relations between the extracellular HSPs and the APCs following internalization of the tumor peptides via CD91 pathway generate both anti-and proinflammatory immune response mediated by T cells [54,55].

As HSPs regulate an extensive component of the immune system, they likely have a role in the optimal function of most immune cells. Importantly, the chaperoning effects of HSPs are necessary for the induction of certain T-cell phenotypes, importantly, Th1, Th2, and Tregs. This presupposes that HSPs are important in the Treg function. To date, the following HSPs have been investigated in relation to Tregs, HSP60, HSP70, and HSP90. HSPs are important in the induction, proliferation, suppressive function, and cytokine production of Tregs (Figure 4).

HSP60 employs the TLR2-signaling pathway in regulating Treg function. TLR2 is expressed on the surfaces of Tregs [56]. Hence, the association between the TLR2 on the Tregs and the HSP stimulates a sequence of events that affect the functional properties of Tregs. Incidentally, increasing levels of HSP60 have correlated with proportional elevations in the intensity of $CD4^+$ $CD25^+$ Treg-directed suppression on the production of TNF-α and IFN-γ [57]. An increase in HSP60 increases ligand binding of the HSP and the TLR2, thus, increasing suppression. This may represent an autoreactive inflammatory response causing autoimmunity [58]. HSP60 also causes an increase in Treg secretion of TGF-β and IL-10 [57]. HSP60 enhances the differentiation of cord blood cells into $CD4^+$ $CD25^+$ *FOXP3*$^+$ Tregs [59]. Similarly, costimulatory signals from p277 also increase the activity of $CD4^+$ $CD25^+$ Tregs [57]. Therapeutic administration of HSP60 increases the presence of nTregs, and this is often correlated with a decrease in atherosclerotic plaques, the generation of Tregs, and an increase in the production of TGF-β [60,61]. The concentration of HSP60 affects Treg suppression and proliferation. Hence, with respect to TLR2 on Tregs, strong ligand binding results in Treg proliferation while relatively low levels or interactions of ligands and TLR2 on the Treg result in an increase in Treg suppression [62].

Equally, HSP70 in Tregs promotes heightened suppressive function in Tregs [63]. HSP70 confers its activity via the TLR4 pathway inducing a surge in the regulatory activities of Tregs. The TLR4-signaling pathway is important in Treg function, and this may be important for *FOXP3* induction and suppression of inflammatory reactions [64]. TLR4 interactions with HSP70 may also augment effector T cell suppression by Tregs as this has been confirmed in coculture experiments with other ligands [65]. Additionally, the type of Tregs present following HSP administration may be dependent on the type of inflammatory response occurring at the time. For example in the mice model of atherosclerosis, immunization with HSP70 produces a significant amount of $CD4^+$ $CD25^+$ *FOXP3*$^+$ Tregs [60]. Similarly, adoptive transfer of HSP70 peptide epitopes such as B29 induces antigen-specific *FOXP3*$^+$ or LAG-3^+ $CD4^+$ $CD25^+$

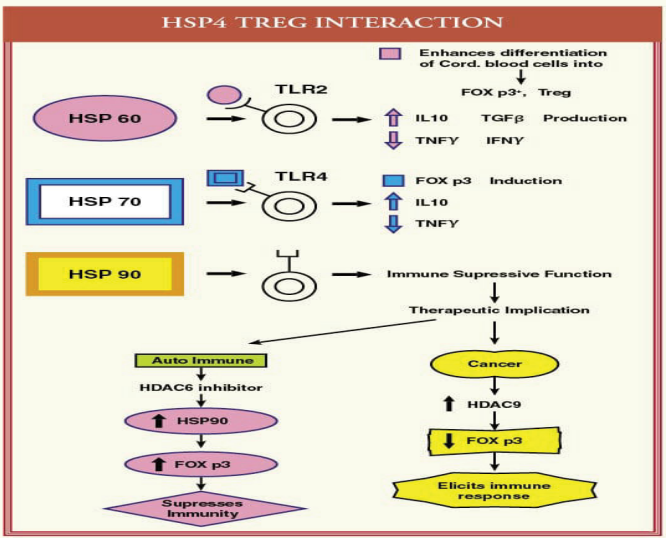

Figure 4. Interaction between HSP and Treg cells.

Tregs that are effective in either aborting or suppressing arthritis in mice [66]. B29 is a highly immunogenic peptide with conserved sequences that are presented to T cells by MHC II molecules. Immunization with HSP70 increases IL-10 producing Tregs [67]. HSP70 derived from Mycobacterium tuberculosis stimulates the proliferation of Tregs by acting through dendritic cells causing a surge in IL-10 while dampening TNF-α release [68]. Additionally, HSP70 has anti-inflammatory properties including down-regulating inflammatory cytokine production, increasing cell and tissue tolerance of cytokine-related cytotoxicity, and influencing the permeability of the epithelial barrier [69].

HSP90 is important for conserving proteins involved in signal transduction, via a multichaperone complex [70]. HSP90 can be regulated by histone deacetylases (HDACs) such as HDAC6, and hypoacetylation of HSP90 occurs in the presence of excessive HDAC6 [71]. HDAC6 belongs to the Class II family of HDACs that are necessary for the removal of acetyl from histones and are found in the nucleus and cytoplasm [72]. In Tregs, the removal of HDAC6 results in the overexpression of HSP90 acetylation resulting in an increase in HSF1-related genes instigating an increase in the suppressive function of Tregs [63]. This may be important in treating patients with colitis. Mice deficient in HDADC6 are more likely to have increased levels of Treg suppression due to the presence of HSP90 and excess *FOXP3* [63]. Similarly, mice deficient in HDAC9 have an increased expression of *FOXP3* [73]. The presence of HDAC9 has been observed to decrease *FOXP3* via deacetylation and incidentally Treg function. HSP70 not only acts via the TLR4 to regulate Tregs but also may inhibit HDAC9 ultimately enhancing the release of Tregs and effective Treg repression [74]. Acetylation is a necessary posttranslational modification process for protein production. Hence, increased acetylation of *FOXP3* may avert ubiquitination, increase its regulatory effects, stability, and promote DNA binding [75]. Therapeutic strategies involving the use of HSPs to enhance the availability of *FOXP3*$^+$ Tregs may be important in autoimmune diseases while in diseases like cancer it may be necessary to inhibit *FOXP3* acetylation [76].

Does Treg expression vary with treatment?

In the various laboratory studies, it had been noted that hyperthermia induces HSP in surviving cells. Through its anti-apoptotic properties and upregulation of immunosuppressive *FOXP3* Tregs, HSPs contribute to the survival and proliferation of the residual tumor cells. This may look counter-productive and highlights the need for achieving complete cytoreduction (CC-0- no residual tumor cell and CC-1-residual tumor deposit < 2.5mm). The intriguing point is that these cells were in ideal growth conditions within the nutrient-rich medium. But in patients undergoing HIPEC, the peritonectomy procedure renders the surviving cells hypoxic and heat augments the penetration and cytotoxicity of chemotherapy agents.

We selected patients with advanced epithelial ovarian cancer with FIGO stage III disease undergoing cytoreductive surgery and HIPEC (n=21 with 7 in each group viz., primary, interval, and secondary). The basic clinical and demographic profile was captured. After the Ethical Committee approval, the study was conducted from June 2017 to June 2020. We included patients with a performance status of ECOG 2 and above with epithelial ovarian cancer treated at IRCH AIIMS and willing for regular follow up till 5 years. After written informed consent, samples were taken from the operated specimen (tumor tissue and surrounding normal tissue). The patients were followed up every 3 months for the first two years, then every six months thereafter.

RNA isolation: The freshly collected tissue samples were stored at -80 °C using Trizol. Then about 1ml of Trizol was added to 50-100 mg of tissue. Then 0.2 ml of chloroform was added per 1 ml of trizol and centrifuged at 12,000 g at 4 °C. The aqueous phase was transferred to a new tube and 0.5 ml of isopropanol was added to each ml of the aqueous phase and centrifuged at 12,000 g at 4º C for 10 min. The white RNA pellet was re-suspended in 1 ml of 75% ethanol and centrifuged at 75000 g for 5 min at 4º C. The supernatant was discarded and the pellet was air-dried and re-suspended in 30 mcl of warm RNAse-free water. The RNA purity and yield were checked by nanodrop.

CDNA synthesis: After RNA isolation, cDNA was synthesized using random hexamers. To 1 µl of RNA sample, 1 µl of random hexamers was added with RNAse free water and heated at 65°C for 5 minutes and incubated on ice for 5 minutes Then 1 µl of ribolock, 2 µl of dNTP mix, 1 µl RevertAid RT, and 4 µl of 5x buffer was added.

PCR: To determine the optimum annealing temperatures, a gradient PCR reaction was set up from 55°C to 65°C. To 1 µl of 1: 5 diluted cDNA, 200 µl of dNTP, 1x buffer 1% BSA, 1% PEG, 1U Taq polymerase were added. Initial denaturation was done at 94°C for 2 minutes. This was followed by 35 cycles of PCR. Each cycle includes denaturation at 94°C for 10 seconds; annealing at 55-65°C for 30 seconds; extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes.

***FOXP3*:** The annealing temperature of 59°C was chosen for *FOXP3* because no non-specific bands were observed at this temperature. The exact size of the *FOXP3* gene product is 350 bp. Since *FOXP3* is GC rich, dimethyl sulfoxide was used in 1.7% concentration to break GC bonds and to reduce the melting temperature of the reaction.

Immunohistochemistry

The formalin-fixed paraffin-embedded tissue blocks were cut at 3-4 micron thickness.IHC for *FOXP3* was performed using the Streptavidin Biotin peroxidase technique on the coated section of tumor tissue from all cases using the Invitrogen monoclonal antibody in 1:200 dilution (clone 236A/E7). Tonsil tissue was taken as a positive control (Table 1). IHC for Th17 cells was done using RORγT antibody (source: Biocare clone ACR3208A) in 1: 100 dilution. Tonsil tissue was taken as a positive control.

Discussion

In our study, there was no statistically significant difference in the expression of *FOXP3* mRNA between the tumor tissue and the surrounding normal tissue in the upfront group (*P*=0.1917) and interval group (*P*=0.7904). However, there was a significant difference observed in the recurrent group (*P*=0.0039). There was a statistical difference in FOX p3 mRNA expression in the tumor tissue among the three groups. (One way ANOVA F-statistic value=10.87837 and p-value= 0.0008) (Figure 5). This reflects the upregulation of immune-suppressive *FOXP3* T regulatory cells in recurrent tumors. There was significant concordance between mRNA and protein expression of *FOXP3* Treg cells in tumor tissue. (Spearmann coefficient is 0.84 and 2-tailed *P*-value=0). It was noted that high levels of *FOXP3* Treg cell expression were

Table 1. Analysis of FOXp3 expression by IHC.

Antibody	Expression pattern
Negative	No immunostaining
Low positive	Stained cells < 30/HPF
High positive	Stained cells >30/HPF

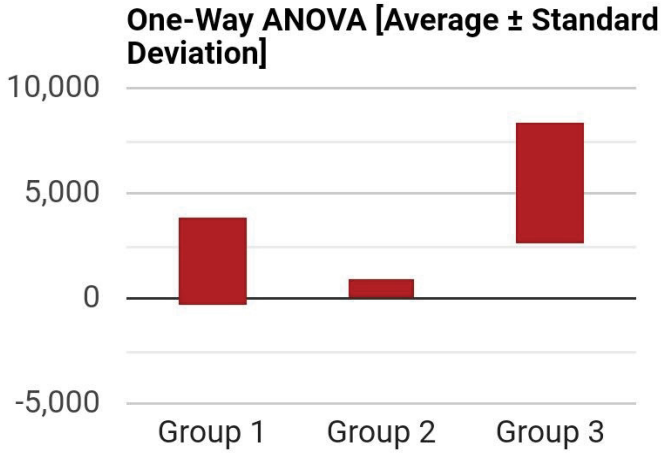

Figure 5. One way ANOVA comparing FOXp3 mRNA expression in three subgroups of ovarian cancer patients undergoing HIPEC (group1-upfront, group 2-interval and group 3- recurrent).

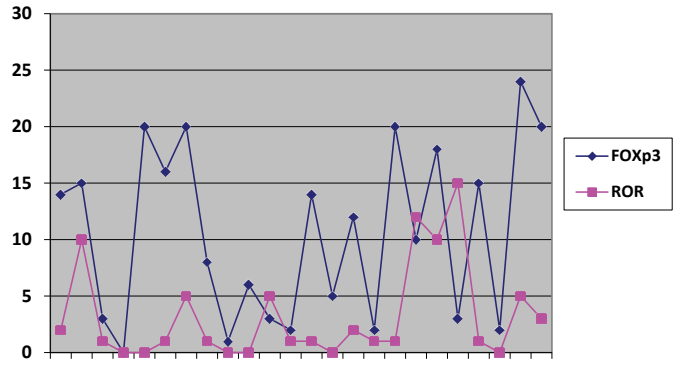

Figure 6. The relation between FOX p3 Treg cells and RORɣ cells based on protein expression by IHC in tumor tissue.

associated with reduced co-expression of RORɣ T-helper cells (Figure 6). *FOXP3* Treg is associated with immune suppression-their overexpression helps tumors to evade immune surveillance. RORɣ T-helper cells Th17 mediate their actions through interleukin 17A and are associated with auto-immune diseases and are found in Tumor-infiltrating lymphocytes. Reduced expression of RORɣ cells helps tumors to evade the immune response.

Conclusion

From this review, we can understand the complex nature of the interaction at a molecular and immunological level in epithelial ovarian cancer patients undergoing HIPEC. Follow-up of these patients with a high level of *FOXP3* cells infiltrating the ovarian tumor tissue, especially those with recurrent tumors will help us to correlate with disease-specific survival and the risk of relapse. This could be used to validate *FOXP3* tumor-infiltrating lymphocyte expression as a novel and independent prognostic factor in epithelial ovarian cancer (EOC). The results of the change in the proportion of *FOXP3* Treg cells in peripheral blood before and after HIPEC is awaited.

Ethics Approval

After the Ethical Committee approval, the study was conducted from June 2017 to June 2020.

Informed Consent

Written informed consent was obtained from the patient for their anonymized information to be published in this article.

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Competing Interests

The authors declare that there is no conflict of interest.

Conflicts of Interests

None

Limitations of the Study

1. Small sample size.
2. Long term follow up is needed for demonstrating survival benefits.

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