Published in final edited form as:

Cancer Immunol Immunother. 2013 May ; 62(5): 839–849. doi:10.1007/s00262-013-1391-0.

Modulation of p38 MAPK signaling enhances dendritic cell activation of human CD4⁺ Th17 responses to ovarian tumor antigen

Martin J. Cannon,

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205, USA

Department of Obstetrics and Gynecology, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205, USA

Hannah E. Goynes,

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205, USA

Pamela J. B. Stone,

Department of Obstetrics and Gynecology, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205, USA

Laura J. MacDonald,

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205, USA

Lindsey E. James,

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205, USA

Everardo Cobos, and

Division of Hematology and Oncology, Texas Tech University Health Sciences Center and Southwest Cancer Treatment and Research Center, Lubbock, TX, USA

Maurizio Chiriva-Internati

Division of Hematology and Oncology, Texas Tech University Health Sciences Center and Southwest Cancer Treatment and Research Center, Lubbock, TX, USA

Martin J. Cannon: CannonMartin@uams.edu

Abstract

The recent finding that Th17 infiltration of ovarian tumors positively predicts patient outcomes suggests that Th17 responses play a protective role in ovarian tumor immunity. This observation has led to the question of whether Th17 cells could be induced or expanded to therapeutic advantage by tumor vaccination. In this study, we show that treatment of ovarian tumor antigen-loaded, cytokine-matured human dendritic cells (DC) with a combination of IL-15 and a p38 MAP

© Springer-Verlag Berlin Heidelberg 2013

Correspondence to: Martin J. Cannon, CannonMartin@uams.edu, mjcannon@uams.edu.

Electronic supplementary material The online version of this article (doi:10.1007/s00262-013-1391-0) contains supplementary material, which is available to authorized users.

Conflict of interest Dr. Cannon is founder of DCV Technologies Inc, a biotechnology company dedicated to the clinical development of dendritic cell vaccines for the treatment of cancer. Dr. Chiriva-Internati is founder of Kiromic Inc, a biotechnology company that seeks to develop therapeutic cancer vaccines. The other authors declare no conflict of interest.

kinase inhibitor offers potent synergy in antagonism of CD4⁺ Treg induction and redirection toward CD4⁺ Th17 responses that correlate with strong CD8⁺ cytotoxic T lymphocyte (CTL) activation. Ovarian tumor antigen-specific CD4⁺ T cells secrete high levels of IL-17 and show reduced expression of CTLA-4, PD-1, and Foxp3 following activation with IL-15/p38 inhibitor-treated DC. We further show that modulation of p38 MAPK signaling in DC is associated with reduced expression of B7-H1 (PD-L1), loss of indoleamine 2,3-dioxygenase activity, and increased phosphorylation of ERK 1/2 MAPK. These observations may allow the development of innovative DC vaccination strategies to boost Th17 immunity in ovarian cancer patients.

Keywords

Ovarian cancer; Dendritic cells; Th17 cells; Regulatory T cells

Introduction

Immunotherapy based on dendritic cell (DC) stimulation of ovarian tumor antigen-specific T cell responses has strong potential as an alternative treatment to prevent disease recurrence or progression after first-line therapy for ovarian cancer, but tumor-associated immunosuppression through recruitment and expansion of CD4⁺ regulatory T cells (Treg) remains a significant barrier to effective treatment. Groundbreaking studies showed that Treg are recruited to ovarian tumors by the chemokine CCL22 (which is highly expressed by ovarian tumors), and that the presence of Treg confers immune privilege and is associated with a poor prognosis and increased mortality [1]. Other investigators have corroborated these observations, showing that high expression of the forkhead box transcription factor foxp3, which is preferentially expressed by CD4⁺ Treg, is an independent prognostic factor for reduced overall survival in ovarian cancer [2], and that a high CD8⁺ T cell/Treg ratio is associated with a more favorable prognosis for this disease [3].

In sharp contrast with the evidence that Treg infiltration is associated with poor outcomes in ovarian cancer, Th17 T cell infiltration correlates with more favorable clinical outcomes [4]. Tumor-infiltrating Th17 cells were positively associated with effector cells and negatively associated with Treg infiltration [4], with the latter relationship arguably being founded on the known reciprocal regulation of Treg and Th17 differentiation [5, 6]. These observations have led to the question of whether Th17 cells could be induced or expanded to therapeutic advantage, either by tumor vaccines or adoptive immunotherapy [7, 8].

Our approach is based on the premise that active immunotherapy, and particularly dendritic cell (DC) vaccination, designed to drive a tumor antigen-specific Th17 T cell response holds the potential to be of clinical benefit for patients with ovarian cancer. DC are remarkable for their plasticity in directing T cell differentiation and effector function, and thus, the key to success may reside in our ability to educate DC to drive ovarian tumor antigen-specific Th17 responses. Several recent studies have indicated that regulation of the p38 and ERK MAPK signal transduction pathways in DC plays a central role in direction of T cell differentiation. Inhibition of MEK 1/2 and ERK MAPK signaling promotes IL-12 production and Th1 T cell responses, whereas inhibition of p38 MAPK increases signal transduction through ERK 1/2 and blocks IL-12 production [9]. At face value, these observations suggest that inhibition of p38 MAPK signaling would be disadvantageous for DC-driven anti-tumor T cell responses, since this would abrogate Th1 responses. However, p38 inhibition promotes differentiation and survival of monocyte-derived DC [10], and p38 inhibition or MEK/ERK MAPK activation restores deficiencies in DC function in myeloma patients [11], suggesting that treatment of DC with pharmacological inhibitors of p38

signaling may confer benefit. Furthermore, p38 MAPK signaling in DC is associated with increased expression of IL-10 and the induction of tolerance in a mouse model of melanoma, thus contributing to the suppression of anti-tumor T cell responses, whereas inhibition of p38 signaling in DC from tumor-bearing mice markedly suppressed expression of IL-10 and restored the capacity of DC to stimulate T cells [12]. Of particular significance, blockade of the p38 pathway can attenuate regulatory T cell induction by DC and enhance the efficacy of DC vaccination [13], whereas blockade of the ERK pathway suppresses DC-driven Th17 responses [14], suggesting that p38 blockade (which enhances ERK phosphorylation) may favor a switch from Treg induction to Th17 differentiation and expansion.

In this study, we show that treatment of ovarian tumor antigen-loaded, cytokine-matured DC with a combination of IL-15 and a p38 MAPK inhibitor (p38i) offers potent synergy in antagonism of Treg induction and redirection toward Th17 responses that correlate with strong CD8⁺ CTL activation. These observations open the door to the development of innovative DC vaccination strategies to boost Th17 immunity in ovarian cancer patients.

Materials and methods

Human subjects

Ovarian cancer patients ($n = 6$) were recruited from patients attending the Women's Oncology clinic in the Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, under an IRB-approved protocol. Blood samples were drawn at the time of surgery. Further blood draws (to a maximum of 5 draws of 70 mL each) were taken at any time up to 1 year following enrollment into the study. Healthy adult volunteers ($n = 3$) also donated blood for this study. Peripheral blood leukocytes (PBL) were recovered by gradient centrifugation over Lymphoprep (Greiner Bio-One), and cryopreserved in liquid nitrogen.

Lymphoblastoid cell lines (LCL)

Epstein-Barr virus-transformed LCL were established from by infection of PBL with the B95.8 strain of EBV, followed by culture in RPMI 1,640 medium supplemented with 3 mM glutamine, 5×10^{-5} M 2-mercaptoethanol (Sigma) and 10 % fetal bovine serum (Valley Biomedical) (RPMI/10) and 1.0 μ g/ml Cyclosporin A (CsA). LCL were fed after 7 days by a half change of RPMI/10 supplemented with CsA. After 14 days, CsA was withdrawn, and LCL were maintained in RPMI/10.

Dendritic cell and T cell culture

For the preparation of DC, PBL were placed in 12-well plates (Costar) at a concentration of up to 5×10^6 /well in DC medium (CellGenix GmbH). After incubation for 2–3 h at 37 °C, non-adherent cells were removed, the adherent cells were rinsed with phosphate-buffered saline (PBS) and 1.5 ml of DC medium was added to each well, plus 800 U/ml GM-CSF (Sargramostim, Genzyme), 500 U/ml IL-4 (CellGenix), 10 ng/ml IL-15 (CellGenix), and 10 μ M p38i (Catalog # 506121, Calbiochem/EMD Chemicals). On days 3 and 5, half the medium was removed and replaced with DC medium plus the same concentration of cytokines and p38 inhibitor. Maturation cytokines (1 μ M/ml PGE₂ (Sigma), 1,000 U/ml TNF (CellGenix), and 500 U/ml IL-1 (National Cancer Institute Biological Response Modifiers Program) were added on day 5.

For stimulation of peptide tumor antigen-specific T cell responses, DC were loaded with 50 μ g/ml of peptide on day 5 (at the time of addition of maturation cytokines) and the DC were harvested 2 days later. The DC were then washed once with DC medium and used for T cell stimulation at a PBL:DC ratio of 30:1. After 7 days, T cells were collected and restimulated with peptide-loaded irradiated autologous DC at a T cell:DC ratio of between 10:1 and 30:1.

T cells were cultured in 10 ml RPMI 1640 medium supplemented with glutamine, 5×10^{-5} M 2-mercaptoethanol and 10 % human AB serum (Valley Biomedical) (RPMI/10 Hu) in 25 cm² tissue culture flasks (Corning). After the second stimulation, CD4⁺ or CD8⁺ T cells were recovered by positive selection with anti-CD4 or anti-CD8 magnetic antibodies (Miltenyi), respectively, yielding populations of at least 95 % purity. During the second and subsequent T cell passages (up to a maximum of 10 restimulations with peptide-loaded DCs), 35 U/ml IL-2 was added to the medium, and the cultures were periodically fed (every 2–3 days) by changing 50–70 % of the medium and addition of fresh IL-2.

Antigen

The hepsin 48–84 peptide was used as a model ovarian tumor antigen for T cell stimulation. Hepsin is a serine protease that is highly overexpressed by ovarian cancer, and is associated with invasion and metastasis [15]. The hepsin 48–84 peptide was selected from combined analysis of DR1, DR4, and DR7 binding motifs, which has a high probability (> 85 %) of predicting degenerate HLA DR-binding epitopes, or multiple DR-binding clusters [16]. Prior identification of a multi-epitope peptide (including HLA class I-restricted CD8⁺ T cell epitopes) from the ovarian tumor antigen stratum corneum chymotryptic enzyme (kallikrein-7) served as a model for this approach [17]. The hepsin 48–84 sequence is QEPLYPVQVSSADARLMVFDKTEGTWRLCSSLRSNAR. DCs loaded with hepsin 48–84 peptide efficiently stimulated CD4⁺ T cell responses from ovarian cancer patients or healthy adults.

Elisa

IL-17 was measured by ELISA, using a commercially available kit (R&D systems), according to the manufacturer's instructions.

Indoleamine 2,3-dioxygenase (IDO) activity

IDO activity was measured as described [18]. Briefly, DC were washed, resuspended in Hanks balanced salts solution (BSS) plus or minus 100 μ M tryptophan, and incubated at 37 °C for 4 hours. Production of kynurenine, the first stable metabolite of tryptophan downstream of IDO, was measured by spectrophotometry. In the first step, 50 μ l of 30 % trichloroacetic acid (TCA) was added to 100 μ l DC supernatant, and the samples were vortexed and centrifuged at 8,000 $\times$ g for 5 minutes. An equal volume of Ehrlich reagent (100 mg P-dimethylbenzaldehyde in 2 ml glacial acetic acid) was added to the supernatant from the TCA precipitation, and the optical density at 492 nm was measured. L-kynurenine production was quantified by reference to a standard curve of defined kynurenine concentrations.

Flow cytometry

The following fluorochrome-conjugated antibodies were used: FITC-anti-CD80, FITC-anti-CD86, PE-anti-CD83, PE-anti-TNF α , FITC-anti-IFN γ , APC-anti-Foxp3, PE-anti-B7-H1, PE-anti-ICOSL (all from eBioscience), APC-anti- β -catenin, FITC-anti-CCR5, APC-anti-CCR7, FITC-anti-CCR4, PE-anti-CXCR4 (all from R&D Systems), AF647-anti-p38^{phos}, PE-anti-ERK 1/2^{phos}, PE-anti-NF-kB p65^{phos} (all from BD Biosciences), FITC-anti-PD-1 (Bio-Legend), and APC-anti-CTLA-4 (BD PharMingen). Foxp3 and CTLA-4 were stained following fixation and permeabilization (with buffers from eBioscience, according to the manufacturer's protocol). NF-kB p65^{phos}, ERK 1/2^{phos}, and p38^{phos} were stained following fixation (BD Cytofix, Cat. No. 554655) and permeabilization (BD Phosflow permeabilization buffer III, Cat. No. 558050).

Intracellular cytokine expression was assayed following antigen stimulation of CD4⁺ T cells. Briefly, LCL were loaded overnight with peptide antigen (50 µg/ml in serum-free DC medium), irradiated (7,500 cGy), and washed once with PBS prior to coculture with responder CD4⁺ T cells. LCL (2.5×10^5 /well) and T cells (5×10^5 to 1×10^6 /well) were placed in 24-well Costar plates and incubated overnight in the presence of Brefeldin A (eBioscience). Intracellular expression of TNFα and IFNγ was detected following fixation and permeabilization (fixation/permeabilization buffer concentrate, Cat. No. 00-5123-43, diluent, Cat. No. 00-5223-56, and permeabilization buffer, Cat. No. 00-8333-56, all from eBioscience).

All samples were analyzed with a FACSCalibur flow cytometer, using CellQuest software (BD Biosciences). Appropriate isotype controls were used throughout.

Multiplex analyses of DC cytokine expression and signaling pathways

DC cytokine expression was determined with a Milliplex MAP human cytokine/chemokine multiplex assay (Millipore, Cat. No. MPXHCYTO-60 K) for the following analytes: IFNγ, IL-IL-1β, IL-6, IL-10, 12(p70), IL-15, TNFα, and MDC (CCL22). Samples were read with a Luminex 100/200 analysis system (Luminex Corp.). DC signaling pathways were analyzed with an 8-plex multi-pathway signaling kit (Milliplex # 48-680, Millipore) for the determination of ERK 1/2, JNK, p38 MAPK, STAT3, STAT5A/B, p70 S6 kinase, CREB, and IκBα phosphoproteins. Samples were read with a Luminex 100/200 analysis system, with unstimulated and activated HeLa cell lysates as internal controls.

Cytotoxicity assays

Cytotoxicity was tested in a standard chromium release assay. Control or peptide antigen-loaded (50 µg/ml) LCL were incubated in serum-free DC medium overnight at 37 °C. Peptide pulsed targets were then labeled with 50 µCi Na₂[⁵¹Cr]O₄ for an additional hour and washed twice with PBS and once with DC medium before use. Target cells were plated at 1×10^4 /well in 96-well round-bottomed plates with effector T cells at the ratios indicated for each assay. Assays were performed in triplicate wells and incubated for 5 hours at 37 °C. Released ⁵¹Cr in the supernatants was measured with a Cobra Auto-Gamma Counter (Packard), and the percentage of target cell lysis was calculated.

Statistics

Where appropriate, data are displayed with error bars representing one standard deviation. Significance was calculated through one-way analysis of variance.

Results

CD4⁺ helper T cell responses and CD8⁺ CTL responses

We tested the ability of IL-15 and/or p38i to enhance DC-driven T cell responses to a model ovarian tumor antigen (serine protease hepsin peptide 48–84). Monocyte-derived DC (cultured with GM-CSF and IL-4, followed by maturation with TNFα, IL-1β, and PGE₂) induced poor CD4⁺ T cell responses, as measured by IFNγ and TNFα expression upon antigen stimulation, and expanded a significant foxp3⁺ T cell population (Fig. 1a). This was not remedied by DC treatment with IL-15 or p38i, but the combination of both agents markedly enhanced effector CD4⁺ T cell responses, and dramatically reduced the frequency of foxp3⁺ CD4⁺ T cells (Fig. 1a). Furthermore, DC treated with both IL-15 and a p38i activated a robust CD8⁺ CTL response, whereas cytokine-matured DC and DC treated with IL-15 or p38i failed to activate a detectable CTL response against autologous tumor antigen-loaded targets. Representative results from a healthy individual are presented (Fig. 1b). IL-15/p38i-treated DC were capable of stimulating tumor antigen-specific CD8⁺ CTL

responses from healthy subjects and ovarian cancer patients, but it should be noted that not all subjects were capable of mounting a CD8⁺ T cell response to hepsin 48–84. The variability is likely attributable to the impact of HLA class I polymorphism on the ability to present CTL epitopes. Tumor antigen-specific CD4⁺ T cell lines derived by stimulation with IL-15/p38i-treated DC expressed high levels of IL-17 upon antigen activation, but CD4⁺ T cell lines stimulated with cytokine-matured DC or DC treated with IL-15 or p38i alone failed to express IL-17 (Fig. 1c). These observations suggest that IL-15/p38i-treated DC redirect tumor antigen-specific CD4⁺ T cell responses away from activation and expansion of Treg responses and toward a pro-inflammatory Th17 response, and that the Th17 response correlates with activation of a strong CD8⁺ CTL response.

Phenotypic profiles of IL-15/p38i-treated DC and responder CD4⁺ T cells

Flow cytometric analysis of accessory molecule expression by cytokine-matured DC, and DC treated with IL-15 and/or p38i revealed that p38i treatment (with or without IL-15) led to reduced expression of CD80 and CD86 costimulatory molecules, and CD83, a marker of DC maturation (Fig. 2a). These results suggest that p38i treatment may abrogate DC maturation and function. However, expression of the B7-H1 (PD-L1) coregulatory molecule was also markedly reduced following p38i treatment of DC, by about 10-fold in terms of mean fluorescence intensity (Fig. 2b). PD-L1 expression was tested with DC from two healthy individuals and four OvCa patients, and p38 inhibition markedly reduced PD-L1 expression in every case. There were no differences observed between healthy individuals and ovarian cancer patients. The mean MFI for PD-L1 expression by cytokine-matured DC was 152, and the mean MFI for PD-L1 expression by IL-15/p38i-treated DC was 38 ($P < 0.001$). In contrast, DC expression of ICOS-L (B7-H2) was conserved under all treatment conditions (Fig. 2b). DC maturation is associated with down-regulation of CCR5 and other receptors that are responsive to chemokines produced at inflammatory sites [19], and increased expression of CCR7, which confers responsiveness to CCL19 and CCL21 chemokines, promoting migration to draining lymph nodes via afferent lymphatics [20]. DC treated with IL-15 and/or p38 MAPK inhibition did not express CCR5 and consistently expressed high levels of CCR7, with p38 inhibition further enhancing CCR7 expression (Suppl. Fig. 1), suggesting that the migratory capacity of DC would be preserved under these treatments.

Multiplex analysis of cytokine expression by DC from four ovarian cancer patients and one normal donor revealed broadly idiosyncratic patterns, without any discernable trends related to DC treatment (Suppl. Fig. 2a–f), with the exception of a possible trend toward reduced IL-12p70 expression (Suppl. Fig. 2c). The chemokine CCL22 was consistently expressed at high levels by DC (Suppl. Fig. 2g), suggesting they possess the capacity to chemo-attract CCR4⁺ T cells (see below).

CTLA-4 expression has been associated with CD4⁺ Treg phenotype and function [21, 22], and PD-1 engagement by PD-L1 can induce T cell anergy and impair anti-tumor immunity [23]. CD4⁺ T cells stimulated with cytokine-matured DC expressed high levels of CTLA-4, whereas IL-15 or p38i treatment of DC was associated with reduced CD4⁺ T cell expression of CTLA-4, particularly following combined IL-15/p38i treatment of DC (Fig. 3, left). CD4⁺ T cell expression of PD-1 was also reduced following stimulation by p38i-treated DC, and to a lesser extent, by IL-15-treated DC (Fig. 3, right).

Ovarian tumor cells and tumor-associated macrophages express CCL22, which promotes infiltration of CCR4⁺ Treg [1], and may potentially drive migration of CD4⁺CCR4⁺ Th17 T cells. In addition, ovarian tumors express CXCL12 (stromal-derived factor 1) [24], which also drives Treg infiltration [25] and may possibly contribute to recruitment of CD4⁺CXCR4⁺ effector T cells. CD4⁺ T cells stimulated with p38i-treated DC showed

enhanced expression of CCR4, relative to CD4⁺ T cells stimulated with cytokine-matured DC, but showed variably lower expression of CXCR4 (Suppl. Fig. 3).

Modulation of DC signal transduction pathways following p38i treatment

To determine the relationship between signaling pathways and DC phenotype and function, phosphorylation of key molecules was investigated by flow cytometry and multiplex analyses. Pharmacologic inhibition of p38 resulted in increased DC accumulation of p38^{phos} and ERK 1/2^{phos} (Fig. 4). Upregulated ERK pathway activity was expected, given that the p38 pathway negatively regulates ERK signaling. Increased levels of p38^{phos} were observed because the pharmacologic inhibitor of p38 used in these experiments blocks downstream phosphorylation of p38 substrates, rather than phosphorylation of p38 itself. Variable changes in p38 and ERK 1/2 phosphorylation were observed in multiplex assays, but the trends supported the flow cytometric analyses (Suppl. Fig. 4a and 4b). The JNK pathway did not show any significant changes in activity following inhibition of p38 signaling (Suppl. Fig. 4c). NF-κB activity, at least as indicated by Rel65 phosphorylation, was diminished following IL-15 treatment, but essentially unchanged following p38 MAPK inhibition (Fig. 4). However, p38 inhibition decreased levels of activation-induced Ser³²-phosphorylated IκBα (Suppl. Fig. 4d), which may be indicative of ubiquitination and proteasomal degradation, resulting in release and nuclear translocation of NF-κB [26, 27]. Phosphorylation of STAT3, STAT5A/B, p70 S6 kinase, and CREB was not altered by treatment of DC with IL-15 and/or p38 inhibition (not shown).

Indoleamine 2,3-dioxygenase (IDO) activity following p38 inhibition

IDO expression by DC drives Treg responses [28] and controls the balance between Treg and Th17 differentiation [29, 30]. IL-15 treatment of DC had a minimal impact on IDO activity, but inhibition of p38 signaling in DC almost totally abolished IDO activity, as measured by production of kynurenine (an immediate downstream catabolite of tryptophan degradation by IDO) (Fig. 5). Statistically significant differences were observed in IDO activity between the p38i-treated DC and DC in which the p38 MAPK pathway was not inhibited ($P < 0.001$). There was also a significant difference in IDO activity between cytokine-matured DC and IL-15-treated DC ($P = 0.001$), but there was no difference between the p38i-treated and IL-15/p38i-treated DC. These observations were highly reproducible between normal individuals and ovarian cancer patients, and suggest that abrogation of IDO activity following p38 signaling may contribute to DC stimulation of Th17 responses.

Discussion

It has become increasingly evident in recent years that the immune system is intimately involved with the pathogenesis of ovarian cancer. Numerous mechanisms of immune suppression have been identified, including Treg infiltration [1], expression of B7-H1 and B7-H4 coinhibitory molecules by tumor cells and tumor-associated antigen-presenting cells and expression of IDO, all of which are associated with poor clinical outcomes for ovarian cancer patients [31–34]. As a counterweight to these observations, clinical studies have pointed to a role for the immune system in limiting disease progression, suggesting that disease outcomes may be strongly influenced by the balance between immune suppression in the tumor microenvironment versus anti-tumor effector mechanisms. CD3⁺ T cell infiltration in ovarian cancer has been associated with prolonged survival, and patients with tumor-infiltrating T cells were more likely to enjoy optimal cytoreductive surgery (itself a positive correlate with disease-free and overall survival), suggesting that T cell immunity may directly limit disease spread [35]. A high CD8⁺ T cell/Treg ratio has also been associated with a more favorable prognosis [31], and most notably, Th17 cell infiltration

correlates with markedly improved overall survival in ovarian cancer [4]. Although the current evidence in ovarian cancer presents a strong case for Th17-based immunotherapy or tumor vaccination, studies in other malignancies, notably gastric and colon carcinoma [36, 37], have indicated a role for the inflammatory and pro-angiogenic properties of IL-17 in tumor development and progression [38–40], suggesting that the pro- or anti-tumor impact of Th17 immunity may be predicated by the local environment in which the response is shaped. Differing modes of analysis may also present apparently conflicting results from clinical studies. For example, Derhovanessian and colleagues reported an inverse relationship between CCR4⁺IL-17⁺ CD4⁺ T cell frequencies in peripheral blood and time to progression in prostate cancer, although no such association was observed for CCR4⁺IL-17⁺ CD4⁺ T cells in the circulation [41]. Further, a second study indicated that a higher frequency of Th17 prostate-infiltrating lymphocytes was associated with slower disease progression [42], suggesting that Th17 immunity in the tumor microenvironment may limit prostate tumor spread.

Notwithstanding these concerns, there has been a steady accumulation of experimental evidence that Th17 cells are capable of mediating powerful anti-tumor immunity. Local induction of Th17 responses has also conferred a survival advantage in a mouse model of pancreatic cancer [43], and tumor growth and pulmonary metastasis was enhanced following injection of the MC38 colon cancer cell line in IL-17-deficient mice [44], again suggesting a protective role for IL-17-expressing T cells. Furthermore, adoptive transfer of Th17 cells to melanoma-bearing mice had greater therapeutic efficacy than transfer of Th1 cells, and Th17 cells activated tumor-specific CD8⁺ T cells that were required for anti-tumor activity [45, 46]. The idea that Th17 cells can promote effective anti-tumor immunity is underlined by a recent report that human Th17 T cells are long-lived effector memory cells with the capacity to mediate effective anti-tumor immunity in collaboration with CD8 T cells [47]. It was further shown that Th17 cells were relatively resistant to apoptosis, and that apoptosis and persistence were regulated by high expression of HIF-1 α , suggesting that Th17 cells may have a survival advantage in the hypoxic tumor microenvironment.

In this report, we show that treatment of human monocyte-derived cytokine-matured DC with the combination of IL-15 and inhibition of p38 MAPK signaling confers the ability to stimulate Th17 CD4⁺ T cell responses associated with strong CD8⁺ CTL responses. Various phenotypic and functional changes were observed (Fig. 6), any or all of which may contribute to Th17 induction. ERK1/2 signaling, which is elevated in p38i-treated DC, has been implicated in DC-driven Th17 responses [14]. The loss of IDO activity may also be critical for Th17 induction, since IDO reportedly plays a pivotal role in the Treg/Th17 balance [28–30]. Reduced DC expression of B7-H1 following p38 inhibition may also limit expansion of Foxp3⁺ CD4⁺ T cells, as B7-H1 has been associated with differentiation of adaptive Foxp3⁺ Treg [48]. In contrast, ICOSL expression on DC was conserved, which may be important, as ICOSL–ICOS signaling has recently been shown to be critical for Th17 responses in humans [49]. In this context, it is also worth noting that p38 inhibition leads to reduced DC expression of CD80 and CD86, potentially leading to reduced costimulation through CD28. At first glance, this may appear to be a drawback for stimulation of effector T cell responses, but CD28 signaling can exert a veto effect on Th17 activation [49, 50], and thus reduced expression of CD80 and CD86 may indirectly favor a Th17 response. Elucidation of the relative contributions of these key pathways in DC stimulation of Th17 responses will require further investigation.

In conclusion, we have shown that treatment of ovarian tumor antigen-loaded, cytokine-matured DC with a combination of IL-15 and a p38 MAPK inhibitor offers potent synergy in antagonism of Treg induction and redirection toward Th17 responses that correlate with strong CD8⁺ CTL activation. These observations may contribute to the clinical development

of innovative DC vaccination strategies for stimulation of Th17 immunity in ovarian cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by funding from the National Institutes of Health (R01 CA 098927), the Congressionally Directed Medical Research Program in Ovarian Cancer (OC 073266) the Arkansas Center for Clinical and Translational Research (NIH UL1RR029884), and the Mary Kay Foundation (009-11). The authors wish to thank Amy Godley and the clinical staff of the Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, UAMS for their invaluable assistance with procurement of blood samples from ovarian cancer patients.

References

1. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burrow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med.* 2004; 10:942–949. [PubMed: 15322536]
2. Wolf D, Wolf AM, Rumpold H, Fiegl H, Zeimet AG, Muller-Holzner E, Deibl M, Gastl G, Gunsilius E, Marth C. The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. *Clin Cancer Res.* 2005; 11:8326–8331. [PubMed: 16322292]
3. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnajatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ, Odunsi K. Intraepithelial CD8⁺ tumor-infiltrating lymphocytes and a high CD8⁺/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA.* 2005; 102:18538–18543. [PubMed: 16344461]
4. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH, Chang A, Coukos G, Liu R, Zou W. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood.* 2009; 114:1141–1149. [PubMed: 19470694]
5. Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol.* 2007; 8:345–350. [PubMed: 17375096]
6. Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, Kolls J, Chang A, Zou W. Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J Immunol.* 2007; 178:6730–6733. [PubMed: 17513719]
7. Munn DH. Th17 cells in ovarian cancer. *Blood.* 2009; 114:1134–1135. [PubMed: 19661273]
8. Cannon MJ, Goynne H, Stone PJB, Chiriva-Internati M. Dendritic cell vaccination against ovarian cancer—tipping the Treg/Th17 balance to therapeutic advantage. *Exp Opin Biol Ther.* 2011; 11:1–5.
9. Jackson AM, Mulcahy LA, Zhu XW, O'Donnell D, Patel PM. Tumour-mediated disruption of dendritic cell function: inhibiting the MEK1/2-p44/42 axis restores IL-12 production and Th1-generation. *Int J Cancer.* 2008; 123:623–632. [PubMed: 18478568]
10. Xie J, Qian J, Yang J, Wang S, Freeman ME 3rd, Yi Q. Critical roles of Raf/MEK/ERK and PI3 K/AKT signaling and inactivation of p38 MAP kinase in the differentiation and survival of monocyte-derived immature dendritic cells. *Exp Hematol.* 2005; 33:564–572. [PubMed: 15850834]
11. Wang S, Hong S, Yang J, Qian J, Zhang X, Shpall E, Kwak LW, Yi Q. Optimizing immunotherapy in multiple myeloma: restoring the function of patients' monocyte-derived dendritic cells by inhibiting p38 or activating MEK/ERK MAPK and neutralizing interleukin-6 in progenitor cells. *Blood.* 2006; 108:4071–4077. [PubMed: 16917008]

12. Zhao F, Falk C, Osen W, Kato M, Schadendorf D, Umansky V. Activation of p38 mitogen-activated protein kinase drives dendritic cells to become tolerogenic in ret transgenic mice spontaneously developing melanoma. *Clin Cancer Res.* 2009; 15:4382–4390. [PubMed: 19549770]
13. Jarnicki AG, Conroy H, Brereton C, Donnelly G, Toomey D, Walsh K, Sweeney C, Leavy O, Fletcher J, Lavelle EC, Dunne P, Mills KH. Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. *J Immunol.* 2008; 180:3797–3806. [PubMed: 18322186]
14. Brereton C, Sutton CE, Lalor SJ, Lavelle EC, Mills KH. Inhibition of ERK MAPK suppresses IL-23-and IL-1-driven IL-17 production and attenuates autoimmune disease. *J Immunol.* 2009; 183:1715–1723. [PubMed: 19570828]
15. Tanimoto H, Yan Y, Clarke J, Korourian S, Shigemasa K, Parmley TH, Parham GP, O'Brien TJ. Hepsin, a cell surface serine protease identified in hepatoma cells, is overexpressed on ovarian cancer. *Cancer Res.* 1997; 57:2884–2887. [PubMed: 9230195]
16. Southwood S, Sidney J, Kondo A, del Guercio M-F, Appella E, Hoffman S, Kubo RT, Chesnut RW, Grey HM, Sette A. Several common HLA-DR types share largely overlapping peptide binding repertoires. *J Immunol.* 1998; 160:3363–3373. [PubMed: 9531296]
17. Bondurant KL, Crew MD, Santin AD, O'Brien TJ, Cannon MJ. Definition of an immunodominant region within the ovarian tumor antigen stratum corneum chymotryptic enzyme. *Clin Cancer Res.* 2005; 11:3446–3454. [PubMed: 15867247]
18. Braun D, Longman RS, Albert ML. A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. *Blood.* 2005; 106:2375–2381. [PubMed: 15947091]
19. Sallusto F, Schaerli P, Loetscher P, Scharniel C, Lenig D, Mackay CR, Qin S, Lanzavecchia A. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol.* 1998; 28:2760–2769. [PubMed: 9754563]
20. Randolph GJ, Sanchez-Schmitz G, Angeli V. Factors and signals that govern the migration of dendritic cells via lymphatics: recent advances. *Springer Semin Immunol.* 2005; 26:273–287.
21. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3⁺ regulatory T cell function. *Science.* 2008; 322:271–275. [PubMed: 18845758]
22. Zheng Y, Manzotti CN, Burke F, Dussably L, Qureshi O, Walker LSK, Sansom DM. Acquisition of suppressive function by activated human CD4⁺CD25⁺ T cells is associated with the expression of CTLA-4 not Foxp3. *J Immunol.* 2008; 181:1683–1691. [PubMed: 18641304]
23. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Ann Rev Immunol.* 2008; 26:677–704. [PubMed: 18173375]
24. Zou W, Machelon V, Coulomb-L'Hermin A, Borvak J, Nome F, Isaeva T, Wei S, Krzysiek R, Durand-Gasselin I, Gordon A, Pustilnik T, Curiel DT, Galanaud P, Capron F, Emilie D, Curiel TJ. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid dendritic cells. *Nat Med.* 2001; 7:1339–1346. [PubMed: 11726975]
25. Wei S, Kryczek I, Edwards RP, Zou L, Szeliga W, Banerjee M, Cost M, Cheng P, Chang A, Redman B, Herberman RB, Zou W. Interleukin-2 administration alters the CD4⁺Foxp3⁺ T-cell pool and tumor trafficking in patients with ovarian carcinoma. *Cancer Res.* 2007; 67:7487–7494. [PubMed: 17671219]
26. Baeuerle PA. The inducible transcription activator NF- κ B—regulation by distinct protein subunits. *Biochim Biophys Acta.* 1991; 1072:63–80. [PubMed: 2018779]
27. Verma IM, Stevenson JK, Schwarz EM, van Antwerp D, Miyamoto S. Rel/NF- κ B/I κ B family: intimate tales of association and dissociation. *Genes Dev.* 1995; 9:2723–2735. [PubMed: 7590248]
28. Chung DJ, Rossi M, Romano E, Ghith J, Yuan J, Munn DH, Young JW. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. *Blood.* 2009; 114:555–563. [PubMed: 19465693]
29. Sharma MD, Hou DY, Liu Y, Koni PA, Metz R, Chandler P, Mellor AL, He Y, Munn DH. Indoleamine 2,3-dioxygenase controls conversion of Foxp3⁺ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood.* 2009; 113:6102–6111. [PubMed: 19366986]

30. Baban B, Chandler PR, Sharma MD, Pihkala J, Koni PA, Munn DH, Mellor AL. IDO activates regulatory T cells and blocks their conversion into Th17-like T cells. *J Immunol.* 2009; 183:2475–2483. [PubMed: 19635913]
31. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, Higuchi T, Yagi H, Takakura K, Minato N, Honjo T, Fujii S. Programmed cell death 1 ligand 1 and tumorinfiltrating CD8 + T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci USA.* 2007; 104:3360–3365. [PubMed: 17360651]
32. Kryczek I, Wei S, Zhu G, Myers L, Mottram P, Cheng P, Chen L, Coukos G, Zou W. Relationship between B7-H4, regulatory T cells, and patient outcome in human ovarian carcinoma. *Cancer Res.* 2007; 67:8900–8905. [PubMed: 17875732]
33. Okamoto A, Nikaido T, Ochiai K, Takakura S, Saito M, Aoki Y, Ishii N, Yanaihara N, Yamada K, Takikawa O, Kawaguchi R, Isonishi S, Tanaka T, Urashima M. Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. *Clin Cancer Res.* 2005; 11:6030–6039. [PubMed: 16115948]
34. Inaba T, Ino K, Kajiyama H, Yamamoto E, Shibata K, Nawa A, Nagasaka T, Akimoto H, Takikawa O, Kikkawa F. Role of the immunosuppressive enzyme indoleamine 2,3-dioxygenase in the progression of ovarian carcinoma. *Gynecol Oncol.* 2009; 115:185–192. [PubMed: 19665763]
35. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med.* 2003; 348:203–213. [PubMed: 12529460]
36. Iida T, Iwahashi M, Katsuda K, Nakamori M, Nakamura M, Naka T, Ojima T, Ueda K, Hayata K, Nakamura Y, Yamaue H. Tumor-infiltrating CD4⁺ Th17 cells produce IL-17 in tumor microenvironment and promote tumor progression in human gastric cancer. *Oncol Rep.* 2011; 25:1271–1277. [PubMed: 21369705]
37. Liu J, Duan Y, Cheng X, Chen X, Xie W, Long H, Lin Z, Zhu B. IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma. *Biochem Biophys Res Commun.* 2011; 407:348–354. [PubMed: 21396350]
38. Murugaiyan G, Saha B. Protumor vs antitumor functions of IL-17. *J Immunol.* 2009; 183:4169–4175. [PubMed: 19767566]
39. Kryczek I, Wu K, Zhao E, Wei S, Vatan L, Szeliga W, Huang E, Greenson J, Chang A, Rolinski J, Radwan P, Fang J, Wang G, Zou W. IL-17⁺ regulatory T cells in the microenvironments of chronic inflammation and cancer. *J Immunol.* 2011; 186:4388–4395. [PubMed: 21357259]
40. Middleton GW, Annels NE, Pandha HS. Are we ready to start studies of Th17 cell manipulation as a therapy for cancer? *Cancer Immunol Immunother.* 2012; 61:1–7. [PubMed: 22086162]
41. Derhovanessian E, Adams V, Hähnel K, Groeger A, Pandha H, Ward S, Pawelec G. Pretreatment frequency of circulating IL-17⁺ CD4⁺ T-cells, but not Tregs, correlates with clinical response to whole-cell vaccination in prostate cancer patients. *Int J Cancer.* 2009; 125:1372–1379. [PubMed: 19533748]
42. Sfanos KS, Bruno TC, Maris CH, Xu L, Thoburn CJ, DeMarzo AM, Meeker AK, Isaacs WB, Drake CG. Phenotypic analysis of prostate-infiltrating lymphocytes reveals T_H17 and T_{reg} skewing. *Clin Cancer Res.* 2008; 14:3254–3261. [PubMed: 18519750]
43. Gnerlich JL, Mitchem JB, Weir JS, Sankpal NV, Kashiwagi H, Belt BA, Porembka MR, Herndon JM, Eberlein TJ, Goedegebuure P, Linehan DC. Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer. *J Immunol.* 2010; 185:4063–4071. [PubMed: 20805420]
44. Kryczek I, Wei S, Szeliga W, Vatan L, Zou W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood.* 2009; 114:357–359. [PubMed: 19289853]
45. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, Gattinoni L, Wrzesinski C, Hinrichs CS, Kerstann KW, Feigenbaum L, Chan C–C, Restifo NP. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood.* 2008; 112:362–373. [PubMed: 18354038]
46. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, Hwu P, Restifo NP, Overwijk WW, Dong C. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity.* 2009; 31:787–798. [PubMed: 19879162]

47. Kryczek I, Zhao E, Liu Y, Vatan L, Szeliga W, Moyer J, Klimczak A, Lange A, Zou W. Human Th17 cells are long-lived effector memory cells. *Sci Transl Med*. 2011; 3 104ra100.
48. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3⁺CD4⁺ regulatory T cells. *Proc Natl Acad Sci USA*. 2008; 105:9331–9336. [PubMed: 18599457]
49. Paulos CM, Carpenito C, Plesa G, Suhoski MM, Varela-Rohena A, Golovina TN, Carroll RG, Riley JL, June CH. The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells. *Sci Transl Med*. 2010; 2 55ra78.
50. Bouguermouh S, Foritn G, Baba N, Rubio M, Sarfati M. CD28 co-stimulation down regulates Th17 development. *PLoS ONE*. 2009; 4:e5087. [PubMed: 19333372]

Fig. 1.

Functional analysis of hepsin 48–84 tumor antigen-specific CD4⁺ T cell lines derived by stimulation with cytokine-matured DC, IL-15-treated cytokine-matured DC, p38i-treated cytokine-matured DC or IL-15/p38i-treated, cytokine-matured DC. **a** CD4⁺ T cell expression of TNF α , IFN γ and Foxp3 following stimulation with antigen-loaded autologous LCL. CD4⁺ T cells cocultured with control LCL did not express TNF α or IFN γ (not shown). Representative results from two healthy subjects are shown (from a total of ten experiments). **b** CD8⁺ CTL responses against antigen-loaded autologous LCL (filled circle) or control LCL (open circle). **c** CD4⁺ T cell IL-17 expression following stimulation with

antigen-loaded autologous LCL. Statistically significant differences were observed between IL-17 expression by the IL-15/p38i DC-derived CD4⁺ T cells and all other CD4⁺ T cell groups (**P* = 0.008). For all groups, CD4⁺ T cells cocultured with control LCL expressed <15 pg/ml IL-17 (not shown). LCL alone did not express IL-17

Fig. 2. Costimulatory molecule expression by mature DC following treatment with IL-15 and/or p38 inhibition. **a** Expression of CD80, CD86, and CD83. **b** Expression of B7-H1 (PD-L1) and ICOS-L

Fig. 3.
CTLA-4 and PD-1 expression by CD4⁺ T cell lines derived by DC stimulation following treatment of DC with IL-15 and/or p38 inhibition

Fig. 4. Phosphorylation of p38, ERK, and NF-κB following treatment of DC with IL-15 and/or p38 inhibition

Fig. 5.

IDO activity in DC. Kynurenine production by cytokine-matured DC, IL-15-treated cytokine-matured DC, p38i-treated cytokine-matured DC or IL-15/p38i-treated, cytokine-matured DC, incubated with or without tryptophan (the IDO substrate). Statistically significant differences in IDO activity were observed between p38i-treated and untreated DC, and also between cytokine-matured DC and IL-15-treated cytokine-matured DC (* $P < 0.001$ for all comparisons)