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Exogenous IL-2 induces FoxP3⁺ Th17 cells in vivo in melanoma patients

Maggie L. Diller¹, Ragini R. Kudchadkar², Keith A. Delman³, David H. Lawson², and Mandy L. Ford⁴

¹Department of Surgery of Emory University

²Department of Hematology and Medical Oncology, Winship Cancer Institute of Emory University

³Department of Surgical Oncology, Winship Cancer Institute of Emory University

⁵Emory Transplant Center of Emory University

Abstract

Introduction—Th17 cells represent a distinct subset of CD4⁺ effector T cells with potent pathogenic qualities, capable of directly mediating tumor cell destruction. IL-2 has frequently been shown to have a negative impact on Th17 differentiation while supporting regulatory T cell (FoxP3⁺CD4⁺, T_{REG}) growth and development in both in vitro models and in vivo animal models. We investigated the effect of in vivo IL-2 on both the Th17 and FoxP3⁺CD4⁺ T cell compartments in a human model of cancer.

Methods—High-dose IL-2 (HDIL-2) was administered at a dose of 720,000 IU/kg to patients with melanoma (n=7) and peripheral blood was collected at baseline and at 24, 48, 72, and 96 hours post-treatment. PBMCs were isolated and underwent intracellular cytokine and extracellular receptor staining for flow cytometry.

Results—We report that HDIL-2 increased both frequencies and absolute numbers of Th17 cells on day 4 of treatment. The administration of HDIL-2 to patients with melanoma increased IL-6 production by peripheral immune cells, a cytokine vital in the downregulation of FoxP3 expression and expansion of the Th17 cell population. Furthermore, we demonstrated that FoxP3⁺CD4⁺ T cells express IL-17 in patients with melanoma undergoing HDIL-2 therapy.

Conclusions—Taken together, our findings indicate that HDIL-2 combined with the conditions of malignancy create an immune environment supportive of Th17 differentiation and that expansion of this compartment may occur via the trans-differentiation of IL-17-secreting FoxP3⁺CD4⁺ T cells.

Corresponding author: Mandy L. Ford, Mandy.Ford@emory.edu, Emory Transplant center, 1365 Clifton Rd. NE, Atlanta, GA 30322, Phone: (855) 366-7989, Fax: (440) 727-3660.

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Authorship

Contribution: M.L.D. and M.L.F. contributed to study concept, design, independent review of all studies, data collection and interpretation, drafting of manuscript and manuscript revision. K.A.D., R.R.K., and D.H.L. contributed to study concept, protocol development, data interpretation, manuscript revision, and approval of final version. All authors read and approved the manuscript.

Keywords

Th17 cells; FoxP3⁺Th17 cells; IL-6⁺ monocytes; high dose IL-2 (HDIL-2); melanoma

Introduction

IL-2 is a critical growth factor for T cells and supports T cell function. Because of its known effects on T helper (Th1 and Th2) and CD8⁺ T cell populations, high dose IL-2 (HDIL-2) has served as a principal means of immunomodulation in the setting of melanoma and renal cell carcinoma since the early 1990s. While the response rate is low (<20%), it has been shown to induce complete and durable responses in 5–10% of patients [1]. Rosenberg et al. subsequently demonstrated that administration of HDIL-2 to immune intact patients with melanoma increased functionally suppressive regulatory T cells (CD25⁺FoxP3⁺CD4⁺, T_{REG}) [2]. While T_{REG} frequencies remained elevated for up to 3 weeks in patients who failed therapy, they declined in those who responded, and the balance between the regulatory and effector T cell compartments appeared to be closely associated with clinical outcomes [3].

Historically, CD4⁺ T cells have been considered helper cells, enhancing the activity of CD8⁺ effector cells. However, recent studies suggest that CD4⁺ T cells may play a more important and direct role in tumor eradication than previously thought [4–6]. Tumor-specific CD4⁺ T cells have been shown to acquire cytotoxic activity and induce an anti-tumor response in vivo in mouse models of B16 melanoma [5, 6]. Furthermore, CD4⁺ T cell-mediated tumor regression was independent of CD8⁺ T cells, B cells, and NK cells [5]. A subset of effector CD4⁺ T cells that secrete the cytokine IL-17 (Th17 cells), have received attention recently for their potent antitumor effects [7, 8]. Th17 cells represent a unique CD4⁺ T cell subset, independent of the Th1 and Th2-promoting transcription factors T-bet, GATA-3, and signal transducers STAT1, STAT4, and STAT6 [9–11]. Instead, STAT3 activation and RORγt expression are critical for the development and differentiation of Th17 cells [10, 12, 13]. Furthermore, Th17 cells do not depend on IL-17 but rather on IL-6 and TGF-β for differentiation [10, 14–16].

TGF-β is important for both the induction and maintenance of FoxP3⁺ T_{REG}s in the peripheral compartment. However, the addition of IL-6 to TGF-β has been shown to downregulate FoxP3 and drive Th17 differentiation, suggesting a reciprocal relationship between regulatory Foxp3⁺CD4⁺ T cells and inflammatory Th17 cells [10, 14]. Studies performed in the setting of IL-2 further support this reciprocity, whereby IL-2 induces expansion of the regulatory T cell compartment and inhibits Th17 cell differentiation [14, 17–21]. However, some studies call into question this dichotomy and have shown that exogenous IL-2 is capable of promoting Th17 expansion, particularly in the setting of autoimmune diseases [22, 23]. Furthermore, there is a growing body of evidence suggesting that FoxP3⁺CD4⁺ T cells may induce or even differentiate into Th17 cells themselves [17, 24]. The current body of literature analyzing the impact of IL-2 on the Th17 and FoxP3⁺CD4⁺ T cell compartments is limited to in vitro treatment of human cells or in vivo animal models. In this study, we aimed to investigate the impact of in vivo administration of

IL-2 in humans on both the Th17 and FoxP3⁺CD4⁺ T cell compartments and interrogated the mechanisms driving Th17 differentiation in the setting of IL-2 in patients with metastatic melanoma.

Materials and Methods

Patient enrollment and sample collection

All patients undergoing HDIL-2 therapy at Emory University Hospital between the years 2013 and 2015 were enrolled in a protocol approved by Emory University's Clinical and Translational Review Committee (CTRC), Department of Surgery, and Institutional Review Board (IRB #00046593) after informed consent was obtained (n=7). All patient samples were acquired during cycle 1 (days 1–5) of course 1 of HDIL-2 treatment. HDIL-2 (aldesleukin; Prometheus, San Diego CA) was administered as an IV infusion over 15 minutes at a dose of 720,000 IU/kg every 8 hours (6 am, 2 pm, 10 pm) and continued for a maximum of 5 days. Peripheral blood was obtained pretreatment and every 24 hours for the duration of HDIL-2 therapy. All samples were drawn between 10 am and 11 am, 4–5 hours after the administration of HDIL-2, to correspond with morning labs. PBMCs were purified from peripheral blood samples via density gradient centrifugation (cell preparation tubes, BD Pharmingen) and cryopreserved at -80 degrees C for future intracellular and extracellular staining and analysis via flow cytometry.

Ex vivo frequency and phenotypic analysis of isolated PBMCs

Standard extracellular staining was performed on PBMCs using the following fluorophore-labeled antibodies: CD3-Alexa700 (Biolegend), CD14/CD20-PacOrange (Invitrogen), CD8-APC-Cy7 (BD pharmingen), CD4-Pac Blue (Biolegend), CD45RA-Qdot655 (Invitrogen), CCR7-PECy7 (Biolegend), CD25-PE (Biolegend). For FoxP3 staining, cells were stained using the FoxP3 staining kit (eBioscience) according to manufacturer's protocol. Frequencies of cell populations and co-signalling molecule expression were determined by flow cytometry.

Ex vivo intracellular cytokine staining

For determination of ex vivo cytokine production, PBMCs were suspended in 1640 RPMI medium supplemented with 10% heat-inactivated FBS (fetal bovine serum), 1% L-glutamine (200 mM), 1% penicillin/streptomycin (100 ×), 1% Hepes (1 M), 1% 2-ME (14.3 M). For T cell stimulation, 1×10^6 PBMCs were placed in a 96 well plate and stimulated for 4 hours at 37 degrees C with a mixture of PMA (Sigma) and ionomycin (Sigma) at a concentration of 1 µg/mL each. For monocyte stimulation, 1×10^6 PBMCs were placed in a 96 well plate and stimulated for 4 hours at 37 degrees C with LPS at a concentration of 1 µg/mL. Brefeldin A (GolgiPlug, BD Biosciences) was added to all cells after 1 hour of stimulation at a concentration of 1 µg/mL. Intracellular staining was performed after fixation and permeabilization according to manufacturer's instructions (BD Biosciences) utilizing fluorophore-labeled antibodies to IFNγ-APC, IL-6-FITC, IL-17-APC-Cy7, IL-10-APC (all antibodies were purchased from Biolegend). Cytokine production was determined via flow cytometry.

Rare event detection

We utilized the following procedures and techniques which have been shown to enhance the detection of rare events via flow cytometry [25]: 1) “no-lyse/no-wash” procedures, 2) the flow cytometer instrument and fluidics were cleaned before each use, 3) we utilized a multi-parameter gating strategy for each cell population of interest with at least one fluorescence parameter for which the rare event was negative (“dump” channel).

Statistics

Statistical analysis was performed using non-parametric analyses including: 2-tailed paired (Wilcoxon signed-rank test) and unpaired (Mann-Whitney U test) procedures, Kruskal Wallis or Friedman tests with post-hoc multiple comparison procedure, and Spearman’s non-parametric assessment of correlation with Prism 5.0 (GraphPad) software. *P* values of less than 0.05 were considered statistically significant.

Results

HDIL-2 induced lymphopenia is associated with an enrichment of CCR7⁺CD3⁺ T cells within the peripheral compartment on day 3 of therapy

To begin our investigation of changes in lymphocyte populations following HDIL-2 therapy, we collected complete blood counts (CBCs) from all patients at days 0, 1, 2, 3, and 4 of treatment. Analysis of absolute lymphocyte count (ALC) demonstrated a decrease from baseline (day 0) at days 2 and 3 (day 0 vs. day 2, *p*= 0.0003; day 0 vs. day 3, *p*= 0.03, Figure 1A, C). Total lymphocyte counts returned to baseline values by day 4 of therapy. In order to investigate changes in the T cell compartment specifically, we performed flow cytometric analyses of PBMCs at days 0, 1, 2, 3, and 4. Absolute numbers of CD3⁺ T cells followed a similar trend with a decrease from baseline occurring at day 3 (day 0 vs. day 3, *p*= 0.004; Figure 1B,D). It is important to note that patient lymphopenia limited the number of samples we were able to analyze at the day 1 and day 2 time points and likely contributes to the lack of statistical significance in CD3⁺ T cell counts between days 0, 1, and 2. To explore potential causes of HDIL-2 lymphopenia, we analyzed CCR7, a chemokine receptor important in the homing of T cells to secondary lymphoid organs [26, 27]. On day 3 of HDIL-2 induced lymphopenia, a larger proportion of cells remaining in the periphery were CCR7⁺ (31% $\pm$ 5% CCR7⁺ vs. 68% $\pm$ 5% CCR7⁺, *p*=0.01; Figure 1E).

Interestingly, reconstitution of T cells at day 4 of therapy was not associated with an increase in CCR7⁺ memory, or “pseudomemory,” T cells as is observed in virally induced lymphopenias or those secondary to therapeutic T cell depletion [28, 29](Figure 1F, G). To further investigate the distribution of naïve vs. memory T cells during HDIL-2 induced lymphopenia and subsequent immune reconstitution, we analyzed CCR7 and CD45RA expression on CD3⁺ T cells during HDIL-2 therapy. Due to the pronounced lymphopenia at days 1 and 2 of treatment, analysis was limited to days 0, 3, and 4. On day 3 of therapy, when CD3⁺ T cell counts were lowest, our results showed a decrease in the central memory (CD45RA⁺CCR7⁺, T_{CM}) compartment (37% $\pm$ 6% on day 0 vs. 22% $\pm$ 7% on day 3, *p*=0.03; Figure 1F, G) and an increase in the effector memory (CD45RA⁺CCR7⁺, T_{EM}) compartment (29% $\pm$ 5% on day 0 vs. 49% $\pm$ 6% on day 3, *p*=0.03; Figure 1F, G). Upon

resolution of HDIL-2 induced lymphopenia (day 4), frequencies within both the T_{CM} and T_{EM} compartments had returned to baseline (Figure 1F,G).

HDIL-2 increased frequency and number of IL-17-producing CD4⁺ T cells on day 4 of therapy in patients with advanced stage melanoma

IL-2 has been shown to differentially affect T cell populations within the human CD4⁺ compartment in vitro: promoting the growth of Th1 cells while negatively impacting Th17 differentiation [21, 23, 30]. In order to more fully investigate the effect of in vivo HDIL-2 on CD4⁺ T cell effector function we analyzed IFN γ , IL-2, and IL-17 production in 6 patients with advanced stage melanoma on days 0 and 4 of HDIL-2 therapy via flow cytometry. While no statistically significant change in the expression of the Th1 cytokines IFN γ and IL-2 (see figure 2A–F) was noted, we observed a statistically significant increase in the frequency of IL-17⁺CD4⁺ T cells (Th17 cells) (0.5% $\pm$ 0.1% on day 0 vs. 1.6% $\pm$ 0.3% on day 4, $p=0.03$; Figure 2G–H) on day 4 of HDIL-2 therapy. Absolute numbers of Th17 cells also increased on day 4 of therapy (6 $\pm$ 2 cells/ μ L on day 0 versus 33 $\pm$ 10 cells/ μ L on day 4, $p=0.03$; Figure 2I). These findings demonstrate that in melanoma patients, IL-2 functions to augment Th17 cells in vivo.

Frequencies of FoxP3⁺CD4⁺ T cells peaked at day 3 of HDIL-2 therapy and preceded peak Th17 cell frequencies and cell counts

Th17 cell differentiation and function is closely related to the development and function of T_{REGs} [31]. It is well documented that HDIL-2 induces expansion of FoxP3⁺ T_{REGs} in patients with melanoma [2]. We therefore sought to investigate the impact of HDIL-2 on the FoxP3⁺CD4⁺ T cell compartment relative to the changes that occur within the Th17 compartment during the course of HDIL-2 therapy. We performed longitudinal analyses of these compartments in melanoma patients treated with high-dose IL-2. Frequencies of FoxP3⁺CD4⁺ T cells increased between days 0 and 3 of therapy (14% $\pm$ 2% on day 0 to 71% $\pm$ 4% on day 3, $p=0.01$, Figure 3A–B). Interestingly, frequencies of FoxP3⁺CD4⁺ T cells decreased between days 3 and 4 of therapy, thus returning closer to baseline (71% $\pm$ 4% on day 3 to 24% $\pm$ 8% on day 4, $p=0.04$, Figure 3A–B). There was no statistically significant change in absolute numbers of FoxP3⁺CD4⁺ T cells between days 0, 3, and 4 (not shown). Consistent with our findings presented above, Th17 cell frequencies peaked on day 4 of therapy (0.6% $\pm$ 0.1% on day 0 to 1% $\pm$ 0.1% on day 3 (ns) to 2% $\pm$ 0.3% on day 4; $p=0.002$, Figure 3C). Th17 cell counts increased significantly between days 0 and 4 (5 $\pm$ 2 cells/ μ L on day 0 to 34 $\pm$ 10 cells/ μ L on day 4, $p=0.006$; Figure 3D) and between days 3 and 4 (5 $\pm$ 0.1 cell/ μ L on day 3 to 34 $\pm$ 10 cells/ μ L on day 4, $p=0.04$; Figure 3D). As shown in figure 3E, frequencies of FoxP3⁺CD4⁺ T cells peaked on day 3 of treatment and preceded peak Th17 cell frequency.

FoxP3⁺CD4⁺ T cells secrete IL-17 and IL-10 on day 3 of HDIL-2 therapy

Seminal studies have revealed that FoxP3⁺CD4⁺ T cells are capable of both inducing and becoming Th17 cells [24]. We have demonstrated that HDIL-2 promotes both FoxP3⁺CD4⁺ and Th17 cell differentiation, with maximum FoxP3⁺CD4⁺ T cell expansion preceding maximum Th17 expansion. We therefore speculated that FoxP3⁺CD4⁺ T cells might acquire the ability to secrete IL-17 in vivo in the setting of HDIL-2. We began our investigation by

interrogating FoxP3⁺CD4⁺ T cell function and phenotype via flow cytometry, analyzing IL-10 and IL-17 expression on FoxP3⁺ and FoxP3⁻ CD4⁺ T cells on day 3 of therapy. Unstimulated (US) samples measured at the day 3 time point were utilized as negative controls (average frequency of IL-10⁺FoxP3⁻CD4⁺ T cells = 0.1% ± 0.005% unstimulated vs. 0.2% ± 0.08% stimulated; average frequency of IL-10⁺FoxP3⁺CD4⁺ T cells = 0.6% ± 0.3% unstimulated vs. 0.9% ± 0.1% stimulated; average frequency of IL-17⁺FoxP3⁻CD4⁺ T cells = 0.4% ± 0.2% unstimulated vs. 0.9% ± 0.4% stimulated; average frequency of IL-17⁺FoxP3⁺CD4⁺ T cells = 1% ± 0.6% unstimulated vs. 2.4% ± 1% stimulated). There was no statistically significant difference between the FoxP3⁻ and FoxP3⁺CD4⁺ T cell compartments with regards to frequency of IL-10-secreting or IL-17-secreting cells (data not shown). Our findings did demonstrate a significant difference in the absolute numbers of IL-10⁺FoxP3⁺CD4⁺ T cells when compared to IL-10⁺FoxP3⁻CD4⁺ T cells on day 3 of therapy (7 ± 4 IL-10⁺FoxP3⁺CD4⁺ T cells/μL vs. 0.4 ± 0.2 IL-10⁺FoxP3⁻CD4⁺ T cells/μL, p=0.02; Figure 4A, B). Our findings also demonstrated an increase in the absolute numbers of IL-17⁺FoxP3⁺CD4⁺ T cells when compared to conventional Th17 cells (IL-17⁺FoxP3⁻CD4⁺ T cells) on day 3 of therapy (11 ± 3 IL-17⁺FoxP3⁺CD4⁺ T cells/μL vs. 3 ± 2 IL-17⁺FoxP3⁻CD4⁺ T cells/μL, p=0.01; Figure 4C,D).

We next sought to determine whether FoxP3⁺CD4⁺ T cells expressed both IL-17 and IL-10, or whether IL-10- and IL-17-secreting FoxP3⁺CD4⁺ T cells represented two distinct cell populations in this system. Unstimulated (US) samples measured at the day 3 time point were utilized as negative controls (average frequency of IL-10⁺IL-17⁺FoxP3⁻CD4⁺ T cells = 0% ± 0 unstimulated vs. 0.06% ± 0.03% stimulated; average frequency of IL-10⁺IL-17⁺FoxP3⁺CD4⁺ T cells = 0.1% ± 0.08 unstimulated vs. 0.8% ± 0.2% stimulated). Our findings demonstrate an increase in the frequency of IL-10⁺IL-17⁺FoxP3⁺CD4⁺ T cells when compared to IL-10⁺IL-17⁺FoxP3⁻CD4⁺ T cells (p=0.01; Figure 4E, F). There appeared a trend towards a greater total number of IL-10⁺IL-17⁺FoxP3⁺CD4⁺ T cells when compared to IL-10⁺IL-17⁺FoxP3⁻CD4⁺ T cells, however this finding was not significant (p=0.06; data not shown). There was no statistically significant difference in frequencies of IL-10⁺IL-17⁺FoxP3⁺CD4⁺ T cells between day 0 and day 3 of therapy (not shown).

HDIL-2 therapy increased frequency of IL-6 producing-monocytes in patients with advanced melanoma

Given the finding that HDIL-2 resulted in a systemic increase in Th17 cells in vivo, we sought to understand the mechanistic basis of these results. Both IL-6 and IL-21 are important in the development and maintenance of Th17 cells [32–34]. We therefore hypothesized that IL-6 and/or IL-21 would be elevated in patients undergoing systemic HDIL-2 therapy. To test this, we performed flow cytometric analysis on 5 patients with advanced stage melanoma on day 3 of HDIL-2 treatment and compared these values to a cohort of patients with similar clinical features prior to treatment (n=4) as well as healthy controls (n=3). Our results demonstrated that HDIL-2 therapy increased IL-6 production by CD14⁺CD3⁻ monocytes in patients with melanoma (36% ± 16 on day 0 to 78% ± 16 on day 3, p=0.03; Figure 5A–B). While there was no significant difference in frequency of IL-6⁺ monocytes between melanoma patients at baseline and healthy controls, there was a statistically significant difference in frequency of IL-6⁺ monocytes between melanoma

patients after 3 days of treatment and healthy controls (24% $\pm$ 27% control vs. 76% $\pm$ 18% day 3, $p=0.01$; Figure 5A–B). To further interrogate the relationship between monocyte-derived IL-6 and frequency of Th17 cells in vivo following HDIL-2, we analyzed absolute numbers of IL-6⁺ monocytes relative to absolute numbers of Th17 cells in each patient at day 3 of therapy. While there appeared a direct correlation between frequency of IL-6-secreting monocytes and frequency of Th17 cells, this was not statistically significant ($p=0.1$; Figure 5C).

With regard to IL-21 production, patients with melanoma demonstrated a trend towards increased frequencies of IL-21⁺CD4⁺ T cells at baseline when compared to frequencies of IL-21⁺CD4⁺ T cells of healthy controls ($p=0.057$; Figure 5F). Frequencies of IL-21⁺CD4⁺ T cells increased significantly after 3 days of HDIL-2 therapy when compared to those of healthy controls (5% $\pm$ 1% control vs. 36% $\pm$ 16% day 3, $p=0.01$; Figure 5D, F). We observed no statistically significant difference in frequencies of IL-21⁺CD4⁺ T cells between day 0 and day 3 of HDIL-2 therapy (Figure 5F). Patients with melanoma also demonstrated a trend towards increased frequencies of IL-21⁺CD8⁺ T cells at baseline when compared to frequencies of IL-21⁺CD8⁺ T cells of healthy controls ($p=0.056$; Figure 5G). Frequencies of IL-21⁺CD8⁺ T cells were significantly higher when compared to control values after 3 days of therapy (3% $\pm$ 1.5% control vs. 44% $\pm$ 14% day 3, $p=0.009$; Figure 5E, G). There was no statistically significant difference between frequencies of IL-21⁺CD8⁺ T cells between day 0 and day 3 of therapy (Figure 5G). There was no statistically significant difference in IL-21 expression on Th17 cells between the two groups (not shown).

Discussion

While IL-2 has been shown to demonstrate a stimulatory effect on the T_{REG} compartment in vivo [2], several studies have demonstrated an inhibitory effect of IL-2 on Th17 differentiation [17, 18]. However, these studies investigating the impact of IL-2 on the Th17 compartment have been limited to in vitro analyses of human cells or animal models. Our findings showed that high doses of systemic IL-2 administered to patients with advanced stage melanoma resulted in increased frequencies and absolute numbers of Th17 cells by day 4 of therapy. As mentioned above, IL-6 plays a pivotal role in the development of Th17 cells [35, 36]. Previously published studies suggest that IL-6 production in this setting is secondary to both the immune environment that accompanies malignancy as well as administration of IL-2 itself. Melanoma tumor lines acquire the ability to spontaneously secrete IL-6 [37], and in vitro studies have shown IL-2 to be a potent inducer of IL-6 production by human monocytes [38]. In vivo studies have confirmed these findings, demonstrating that patients with malignant melanoma have elevated serum IL-6 levels at baseline compared to healthy controls, and that plasma levels increase with HDIL-2 therapy [39]. Our findings now link these previous results, demonstrating that HDIL-2 functions in vivo to increase the frequency of circulating IL-6⁺ monocytes, resulting in an immune environment favorable for the differentiation and expansion of IL-17-secreting Th17 cells.

It is interesting to note that HDIL-2 increased both Th17 cell frequency and absolute cell numbers. Our findings demonstrated that HDIL-2 induced an early expansion of FoxP3⁺CD4⁺ T cells and that this population of cells was capable of secreting IL-17.

Furthermore, we showed that within the FoxP3⁺CD4⁺ T cell compartment a small but significant proportion of IL-10 secreting FoxP3⁺CD4⁺ T cells also secreted IL-17 (1%). We therefore hypothesize that HDIL-2 induced expansion of the Th17 compartment may occur via a FoxP3⁺CD4⁺ T cell intermediary (Figure 6). In support of our hypothesis, studies have shown that FoxP3⁺ T_{REG}s secrete TGF-β, a growth factor that along with IL-6 induces the differentiation of naïve T cells into Th17 cells, suggesting that regulatory T cells themselves may support Th17 differentiation [15, 40, 41]. A recent in vitro study demonstrated that both CD25⁺ and CD25⁻FoxP3⁺CD4⁺ T cells are not only inducers of Th17 cells, but are themselves capable of secreting IL-17 [24]. Importantly, trans-differentiation between CD25⁻FoxP3⁺CD4⁺ T cells and Th17 cells did not depend on TGF-β but instead required the presence of IL-6 [15]. It is important to note that our study investigated FoxP3⁺CD4⁺ T cells rather than CD25⁺FoxP3⁺CD4⁺ T cells. Furthermore, while a small percentage of FoxP3⁺CD4⁺ T cells secreted IL-10, suggestive of a regulatory phenotype, the suppressive capacity of this cell population was not fully elucidated by the experiments presented herein. As such, FoxP3⁺CD4⁺ T cells presented in this manuscript cannot be decidedly identified as “T_{REG}s.” Nevertheless, the findings presented above suggest potential trans-differentiation between FoxP3⁺CD4⁺ T cells and Th17 cells, contributing to the observed increase in both Th17 cell frequency and number in the setting of HDIL-2.

IL-21 may also play an important role in modulating the balance between FoxP3⁺CD4⁺ T cells and effector Th17 cells within this system. IL-21 has been shown to downregulate FoxP3, impair the suppressive capacity of T_{REG}s and also induce Th17 differentiation [42–44]. We found that melanoma patients treated with HDIL-2 demonstrated higher frequencies of IL-21⁺ T cells within both the CD4⁺ and CD8⁺ compartments when compared to healthy controls. While FoxP3 expression peaked at the same time as IL-21 expression (day 3 of therapy), in vitro studies have shown that IL-21 mediated inhibition of FoxP3 RNA occurred 12–24 hours after exogenous administration of IL-21 [44]. Thus, we hypothesize that the downstream effects of circulating IL-21 on both FoxP3 expression and Th17 expansion may occur in a delayed fashion (i.e. 12–24 hours later). This is consistent with our findings whereby FoxP3 expression decreased and Th17 cells increased on day 4 of therapy. Furthermore, IL-21R ligation has been shown to further increase IL-21 production via a positive feedback loop [34, 45]. While we were unable to measure IL-21 expression beyond the day 3 time point, it is possible that IL-21 would have continued to increase over the course of HDIL-2 therapy, further potentiating the Th17 phenotype.

Interestingly, the observed increase in cytokine producing cells by CD4⁺ T cells was specific to IL-17, as there was no statistically significant change in the frequency or total numbers of IFNγ⁺ or IL-2⁺ CD4⁺ T cells within the peripheral blood. However, Th1 and Th17 cells exhibit different homing properties which may in part explain this phenomenon. While both Th1 and Th17 cells express CCR2, a chemokine involved in the trafficking of tumor infiltrating lymphocytes (TILs) specifically to melanoma lesions [46], Th1 cells also express CCR5 whereas Th17 cells do not [47]. Importantly, systemic IL-2 has been shown to induce inflammatory changes within the tumor itself, leading to the upregulation of CCR5 ligands specifically [48]. Therefore, our analysis may have failed to capture a portion of Th1 cells present within the tumor microenvironment.

It is interesting to note the marked lymphopenia that occurred early in the course of HDIL-2 therapy and its unique recovery pattern, which differs from other means of pharmacologic T cell depletion. It is well-documented that administration of HDIL-2 increases vasopermeability via direct effects on microvascular endothelial cells [49]. While not included in our analysis, increases in adhesion molecules and lymphocyte rolling as seen in virally-induced lymphopenia may also be a contributing factor in this setting [50]. HDIL-2 induced lymphopenia is likely secondary to a variety of mechanisms and allows for migration of cells in to and out of peripheral tissues and lymphoid organs. Because of this cellular flux, it is important to note that studies relying on peripheral blood sampling may fail to capture changes occurring on T cells within other compartments.

As noted in Table 1, there was significant heterogeneity in individual treatment schedules, and this serves as an important point for discussion. Samples were drawn every 24 hours during the first 5 days (cycle 1) of treatment. However, due to scheduling breaks and/or early termination secondary to toxicity, patients received their final doses of HDIL-2 anywhere between the day 2 and day 4 time points. In spite of this, the results presented above demonstrate a uniform temporal response in both absolute cell counts and cell frequencies to HDIL-2. This may suggest that a single bolus of HDIL-2 induces programmed changes within the immune environment in vivo. As such, the resulting immunophenotype depends on length of time from initial exposure (i.e. 4 days) and is largely independent of subsequent dosing. Additional experiments may offer a more definitive conclusion and prove valuable in an era of expanding immunotherapies and combination regimens.

Along with the limitations noted above, there are additional caveats regarding the interpretation of our results, particularly small patient sample size and patient lymphopenia. While HDIL-2 continues to be used in oncologic practice today, there are only a few centers in the country equipped to manage this treatment regimen and few patients are candidates for HDIL-2 given its significant toxicity profile. The lymphopenia described above further limited our ability to complete comprehensive analyses at all time points collected. Additionally, many of the cell subsets presented in this manuscript are relatively rare events. Future analysis of these populations could benefit from the use of positive selection techniques recently employed in studying low frequency populations (i.e. naïve antigen-specific T cells) [51]. It is important to note that this study did not investigate changes occurring on melanoma-antigen specific T cells or tumor infiltrating lymphocytes (TILs). As such, drawing conclusions regarding our findings and their potential impact on the tumor microenvironment is more difficult.

Regardless, analysis of the immune changes occurring in patients following HDIL-2 therapy afforded us a unique opportunity to observe aspects of the fundamental biology of human Th17 cells and their response to IL-2 in vivo. HDIL-2 has been shown to increase FoxP3⁺ T_{REG}s in patients with melanoma [2], highlighting a potential mechanism for treatment failure. Here, we showed that HDIL-2 administered in the setting of IL-6 increased Th17 cells in vivo. Given previous findings demonstrating the capacity for FoxP3⁺CD4⁺ T cell and Th17 cell inter-conversion, it is interesting to speculate that inflammation (systemic or within the tumor microenvironment) could drive FoxP3⁺ T_{REG}s into IL-17 secreting cells, thus promoting tumor regression. In support of this hypothesis, we observed a single patient

within the studied cohort who demonstrated a complete response to treatment, and this was associated with a high Th17:T_{REG} ratio within the peripheral blood [52]. Therefore, understanding the mechanisms by which Th17 cells expand and interrelate with the FoxP3⁺CD4⁺ T cell compartment is clinically relevant both in the setting of HDIL-2 and other immunomodulatory regimens. These findings highlight several potential therapeutic targets that will allow clinicians to tailor a patient's immune environment in order to bring about the desired clinical response to a given treatment strategy.

In summary, we found that HDIL-2 increased Th17 cells in patients with advanced stage melanoma. Administration of HDIL-2 in the setting of malignancy resulted in an immune environment rich in IL-6 and IL-21-secreting immune cells in vivo, which may lead to expansion of the Th17 compartment via the trans-differentiation of FoxP3⁺CD4⁺ T cells into IL-17 secretors.

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Abbreviations

HDIL-2	high dose IL-2
T_{REG}	regulatory T cell
CBC	complete blood count
ALC	absolute lymphocyte count
CR	complete responder
NR	non-responder
TIL	tumor infiltrating lymphocyte
IFN	interferon
LDH	lactate dehydrogenase

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Figure 1. HDIL-2 induced lymphopenia is associated with an enrichment of CCR7⁻CD3⁺ T cells within the peripheral compartment on day 3 of therapy

A. Representative flow plots from a single patient demonstrate a reduction in frequency of lymphocytes early in the course of HDIL-2 therapy. **B.** Representative flow plots from a single patient demonstrate a reduction in frequency of CD3⁺ T cells early in the course of HDIL-2 therapy. **C.** Analysis via one-way ANOVA with multiple comparisons test demonstrates a reduction in total lymphocyte counts during days 2 and 3 of therapy ($p=0.0003$, 0.03 , respectively). **D.** Analysis via one-way ANOVA with a multiple

comparisons test demonstrates a reduction in CD3⁺ T cell counts on day 3 of therapy (p=0.004). **E.** Analysis via two-way ANOVA with a multiple comparisons test demonstrates an increase in frequency of CCR7⁻CD3⁺ T cells on day 3 of therapy (p=0.01). **F.** Representative flow plots demonstrate changes in the T cell subsets (CD45RA⁺CCR7⁺ naïve, CD45RA⁻CCR7⁺ T_{CM}, CD45RA⁻CCR7⁻ T_{EM}, and CD45RA⁺CCR7⁻ T_{EMRA}). **G.** Analysis via two-way ANOVA with a multiple comparisons test demonstrates a decrease in the T_{CM} compartment and an increase in the T_{EM} compartment between days 0 and 3 of therapy (p=0.03 and 0.03, respectively). Frequencies within both compartments return to near baseline on day 4.

Figure 2. HDIL-2 increased frequency and number of IL-17-producing CD4⁺ T cells on day 4 of therapy in patients with advanced stage melanoma

A. Representative flow plots from two patients demonstrate varied IFN γ production by CD4⁺ T cells in response to HDIL-2 therapy. **B.** Non-parametric analysis of matched samples (Wilcoxon test) demonstrates varied changes in the frequency of IFN γ -producing CD4⁺ T cells during HDIL-2 therapy. **C.** Non-parametric analysis (Wilcoxon test) demonstrates varied changes in the total number of IFN γ -producing CD4⁺ T cells during HDIL-2 therapy. **D.** Representative flow plots from two patients demonstrate varied IL-2

production by CD4⁺ T cells in response to HDIL-2 therapy. **E.** Non-parametric analysis (Wilcoxon test) demonstrates varied changes in the frequency of IL-2-producing CD4⁺ T cells during HDIL-2 therapy. **F.** Non-parametric analysis (Wilcoxon test) demonstrates varied changes in the total number of IL-2-producing CD4⁺ T cells during HDIL-2 therapy. **G.** Representative flow plot from a single patient demonstrates increased IL-17 production by CD4⁺ T cells in response to HDIL-2 therapy. **H.** Non-parametric analysis (Wilcoxon test) demonstrates increased frequencies of IL-17 production by CD4⁺ T cells in response to HDIL-2 therapy (p=0.03). **I.** Non-parametric analysis (Wilcoxon test) demonstrates an increase in total numbers of IL-17⁺CD4⁺ T cells in response to HDIL-2 therapy (p=0.03).

Figure 3. Frequencies of FoxP3⁺CD4⁺ T cells peaked on day 3 of HDIL-2 therapy and preceded peak Th17 cell frequencies and cell counts

A. Representative flow plot demonstrates changes in FoxP3 expression on CD4⁺ T cells over the course of HDIL-2 therapy. **B.** Analysis via one-way ANOVA with a multiple comparisons test demonstrates peak frequencies of FoxP3⁺CD4⁺ T cells occurring on day 3 of therapy with a return to near baseline on day 4 of therapy (p=0.01 and 0.04, respectively). **C.** Analysis via one-way ANOVA with a multiple comparisons test demonstrates an increase in the frequency of IL-17⁺CD4⁺ T cells on day 4 of treatment (p=0.002). **D.** Analysis via

one-way ANOVA with a multiple comparisons test demonstrates an increase in the absolute number of IL-17⁺CD4⁺ T cells from baseline on days 3 and 4 of treatment (p=0.006 and 0.04, respectively). **E.** Peak FoxP3⁺CD4⁺ T cell frequency preceded peak Th17 cell frequency.

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Figure 4. FoxP3⁺CD4⁺ T cells secrete IL-17 and IL-10 on day 3 of HDIL-2 therapy

A. Representative flow plots from unstimulated samples (US) and stimulated samples demonstrate IL-10 expression on FoxP3⁻CD4⁺ T cells and FoxP3⁺CD4⁺ T cells on day 3 of HDIL-2 therapy. **B.** Non-parametric analysis of unmatched samples (Mann Whitney test) demonstrates a higher absolute number of IL-10⁺ T cells within the FoxP3⁺CD4⁺ T cell compartment compared to FoxP3⁻CD4⁺ compartment on day 3 of therapy ($p=0.02$). **C.** Representative flow plots from unstimulated samples (US) and stimulated samples demonstrate IL-17 expression on FoxP3⁻CD4⁺ T cells and FoxP3⁺CD4⁺ T cells on day 3 of

HDIL-2 therapy. **D.** Non-parametric analysis (Mann Whitney test) demonstrates a higher absolute number of IL-17⁺ T cells within the FoxP3⁺CD4⁺ T cell compartment compared to FoxP3⁻CD4⁺ compartment on day 3 of therapy (p=0.01). **E.** Representative flow plots from unstimulated (US) and stimulated samples demonstrate co-expression of IL-17 and IL-10 on FoxP3⁻CD4⁺ T cells and FoxP3⁺CD4⁺ T cells. **F.** Non-parametric analysis (Mann Whitney test) demonstrates a higher absolute number of IL-10⁺IL-17⁺ T cells within the FoxP3⁺CD4⁺ T cell compartment compared to FoxP3⁻CD4⁺ T cell compartment on day 3 of therapy (p=0.01).

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Figure 5. HDIL-2 therapy increased frequency of IL-6- producing monocytes in patients with advanced melanoma

A. Representative flow plot demonstrates frequency of IL-6⁺CD14⁺CD3⁻ monocytes in a healthy donor (HD), and at days 0 and 3 of HDIL-2 treatment. **B.** Analysis via one-way ANOVA with a multiple comparisons test demonstrates an increase in IL-6 production by peripheral monocytes after 3 days of HDIL-2 therapy when compared to control and day 0 values ($p=0.01$ and 0.03 , respectively). **C.** Analysis via a non-parametric assessment of correlation (Spearman's test) suggests a direct correlation between absolute number of

IL-6⁺CD14⁺CD3⁻ monocytes on day 3 of therapy and the absolute number of Th17 cells on day 3 of therapy (ns, p=0.1). **D–E.** Representative flow plots demonstrate frequencies of IL-21⁺CD4⁺ T cells (D) and IL-21⁺CD8⁺ T cells (E) in a healthy donor and in a patient with melanoma at days 0 and 3 of treatment. **F.** Analysis via one-way ANOVA with a multiple comparisons test demonstrates an increase in the frequency of IL-21⁺CD4⁺ T cells between healthy controls and patients with melanoma after 3 days of HDIL-2 therapy (p=0.01). **G.** Analysis via one-way ANOVA with a multiple comparisons test demonstrates an increase in the frequency of IL-21⁺CD8⁺ T cells between healthy controls and patients with melanoma after 3 days of HDIL-2 therapy (p=0.009).

Figure 6. Mechanism of HDIL-2 induced Th17 differentiation

In vivo HDIL-2 induces Th17 differentiation via a FoxP3+CD4+ T cell pathway and is dependent on peripheral IL-6.

Table 1

Patient characteristics

Normal lactate dehydrogenase (LDH) = 140–280 U/L. M category: M1a = Metastasis to skin, subcutaneous tissue, or lymph nodes with a normal LDH level; M1c = Metastasis to any other organs or distant spread to any site along with an elevated blood LDH level. Response: NR = non-responder, CR = complete responder.

Mel #	BRAF V600E Mutation	Prior TX with IFN	LDH	M category	CNS metastases	Doses*	Breaks in schedule	Response
Mel 004	Positive	Yes	158	M1a	No	6	0	NR
Mel 005	Positive	No	187	M1c	Yes	6	0	NR
Mel 011	Positive	No	167	M1a	No	6	2	CR
Mel014	Positive	No	183	M1a	No	7	1	NR
Mel 021	Negative	Yes	122	M1c	No	10	0	NR
Mel 022	Negative	No	180	M1a	No	7	1	NR
Mel 026	Positive	No	407	M1c	Yes	5	1	NR

* All doses were administered during cycle 1 (days 1–5) of course 1 of HDIL-2 therapy.