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Lenalidomide Increases Peripheral T-regulatory-Phenotype Cells in

Patients with AIDS - associated Kaposi Sarcoma

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Semi Han

Committee in charge:

    Professor Jack Bui, Chair
    Professor Li- Fan Lu, Co-chair
    Professor James W. Golden

2012

The thesis of Semi Han is approved, and is acceptable

in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2012

DEDICATION

My dedication goes to my loving parents and brother,

Jin Sook Han, Soo Kang Han, and Seong Hee Han

for showing boundless support and adoration towards their

only daughter and sister.

I admire their courage and persistence and wish dearly to follow their footsteps.

Also, to Bui lab members whom I feel blessed to be around.

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ABSTRACT OF THE THESIS

Lenalidomide Increases Peripheral T-regulatory-Phenotype Cells in

Patients with AIDS - associated Kaposi Sarcoma

by

Semi Han

Master of Science in Biology

University of California, San Diego, 2012

Professor Jack Bui, Chair
Professor Li-Fan Lu, Co-chair

Kaposi sarcoma (KS) is the most common malignancy in patients with

human immune-deficiency virus (HIV) and presents a treatment conundrum

because of its variable natural history. Lenalidomide is a drug that has immune-

modulatory, anti-neoplastic, and anti-angiogenic properties. It is used in the

treatment of myelodysplastic disease and multiple myeloma, but its *in vivo* effects on peripheral blood immune activity is not fully explored. In this preliminary report from a Phase I/II trial of lenalidomide in the treatment of AIDS-associated KS, we found that lenalidomide increased the number and percentage of T-regulatory-phenotype cells in the peripheral blood. In patients with measurable regression of KS lesion size as well as number of lesions, there was also an observable accumulation in FOXP3-expressing cells in the KS lesion. We speculate from these findings that lenalidomide may be useful in the treatment of KS in part because of its anti-angiogenic activity, which is correlated with boosting T-regulatory cell frequency in the peripheral blood.

INTRODUCTION

Kaposi sarcoma (KS) is a multifocal angioproliferative tumor of vascular origin characterized by infection with the oncogenic human herpes virus 8 (HHV-8), also known as KS herpes virus (KSHV). [1] Physiological manifestations can range from local cutaneous lesions to a more severe form involving tumor dissemination to visceral organs. [2] The disease presents three distinct stages that are described as flat patch, plaque, or nodule, each stage progressively becoming more and more complex and malignant. [2] Local cutaneous lesions are often benign, however, once disseminated to the viscera KS could result in organ dysfunction and mortality. Most common extracutaneous involvement sites are lung, gastrointestinal tract, and lymph nodes and the lesions can lead to hemorrhage and intussusception in a serious case of gastrointestinal KS. [3]

Four different epidemiological forms of KS include classic KS found in elderly men of Mediterranean descent, iatrogenic KS usually caused by transplantation, an endemic African KS, and Acquired Immune Deficiency Syndrome (AIDS)-associated KS (AIDS-KS). [4] Of the types mentioned AIDS-KS exhibits the most aggressive tumor development. Perhaps such phenomenon is expected due to the fact that co-infection with HIV is known to exacerbate the oncogenicity and angiogenesis of HHV-8 infected KS cells. For example, HIV viral protein Tat 1 can synergize with basic fibroblast growth factor (bFGF) produced by KS cells to facilitate KS growth and mimic extracellular matrix proteins to

sensitize cells to bFGF, which explain AIDS-KS's aggressiveness compared to classical KS lacking Tat 1 protein. [5]

Despite multiple therapeutic options for the treatment of AIDS-KS, 50% of patients with AIDS-KS never achieve full remission and the disease remains incurable. [4,6] Even with highly active antiretroviral therapy (HAART)'s success in reducing the number of new diagnoses, AIDS-KS remains as one of the most frequent malignancies seen in individuals infected with human immunodeficiency virus (HIV)-1. [7] A recent report of CD4 counts at AIDS-KS diagnoses over the course of HIV endemic shows greater proportion of KS occurrence at a higher CD 4 counts (≥ 350 cells/mm^3) in the post-HAART era.[8] It is possible that KS cells in HIV$^+$ patients treated with HAART with normal CD4$^+$ T cell counts may have undergone "cancer immunoediting", a process by which the immune system sculpts the tumor cell repertoire leading to the outgrowth of tumor cell variants that have reduced immunogenicity.[9] In this case, HAART alone would not be sufficient to avoid KS development. Thus, efforts to boost antitumor immunity or augment the immunogenicity of KS cells in combination of antiviral treatment may be useful in the treatment of AIDS- KS.

HHV-8 is the etiologic agent that mainly infects endothelial cells. HHV-8 disrupts the immune system by alternating between latent and lytic state along with HIV. Yet, latent phase is detected in more than 90% of infected cells [10], indicating that latently infected viral proteins such as latency associated nuclear antigen (LANA), viral cyclin D (v-cyc D), Kaposin A, and viral Fas-associated death domain interleukin 1B converting enzyme (FLICE) inhibitory protein (vFLIP)

are key players in disease manifestation. [11] Among those oncogenic proteins, LANA-1 is most prominent for its contribution to pathogenesis as well as being the diagnostic agent of KS lesion. LANA-1 is involved in viral episomal maintenance, desensitization of TGF-β receptor type II, and inhibition of apoptosis via inactivation of tumor suppressor proteins, p53 and pRB.[12,13]

Lenalidomide (LEN; CC-5013, Revlimid®, Celgene Corporation) is a functional and structural analogue of Thalidomide (Thal) that is approved by the Food and Drug Administration (FDA) to treat hematological malignancies like multiple myeloma (MM) and myelodysplastic syndromes associated with 5q chromosome deletion.[14] Both drugs belong to a family of drugs known as immunomodulatory drugs (IMiDs). IMiDs are reported to possess a versatile set of mechanisms that is beneficial in combating solid and hematological tumors through immunomodulatory and anti-angiogenic properties. Moreover, there is strong evidence that IMiDs can directly induce anti-tumorigenic effect via promoting cell growth arrest and caspase-dependent apoptosis of tumor cells. [15] LEN participates in anti-inflammatory activity by inhibiting the synthesis of tumor-necrosis factor – alpha (TNF-α), which is a pro-inflammatory cytokine that up-regulates the expression of endothelial integrin.[16] Altering inflammatory state such as TNF-α can abrogate the formation of nascent blood vessels that is considered the hallmark of angiogenic and metastatic tumor. LEN also inhibits production of other pro-inflammatory cytokines including interleukin (IL)-1b, IL-6, and IL-12 while increasing the production of anti-flammatory cytokine IL-10. [14]

Co-stimulation of T cells is crucial in generating a prolonged and enhanced cytolytic activity that protects the immune system against invasive or aberrant cells. Previous in vitro studies showed that LEN intensifies T cell clonal proliferation by enhancing the initial T- cell activation and overcoming T cell inhibitory activity of CTLA-4.[14] This stimulation capacity is non-discriminatory and can mediate Th1 type anti-tumor immunity by activating helper CD4^{+} and killer CD8^{+} T cells. Such immunomodulatory activity may be beneficial in AIDS-KS patients whose frequency and function of circulating HHV-8 specific CD8^{+} cells are greatly reduced. [17]

As down-stream effect of T cell activation, IMiD increases secretion of IFN-γ and IL-2 from T cells that subsequently trigger natural killer (NK) cell activity. [18] As part of the innate system, NK cells, defined as CD56^{+} in humans[19], serve a pivotal role in protecting against both virally infected and cancerous cells. Because of its compelling protective capability in the setting of viral infection, which was confirmed using herpesvirus and HIV[19], it was imperative to investigate whether LEN's immune activation would induce a significant enhancement on NK cell mediated killing of the KS cells. Previous reports from in vitro study with multiple myeloma (MM) cell line treated with LEN noted a 1.2-1.3 fold increase in CD56^{+} NK cell frequency[18] and others have reported induction of antibody-dependent cell-mediated cytotoxicity (ADCC) of NK cells on solid tumor cells from LEN treatment, which mechanism is dependent on NKG2D/NKG2D ligand interaction. [20]

A great deal of evidence vouches for LEN's immunomodulatory and anti-angiogenic property as mentioned above, However, those claims are highly theoretical and bona fide in vivo conclusions are lacking. In order to answer the outstanding questions regarding LEN's clinical efficacy, our phase I/II clinical trial of LEN initially sought to reveal the impact on NK cell peripheral blood frequency and cytotoxicity, and T cell response, as well as the anti-angiogenic and anti-metastatic effects of LEN. Our experimental design was the first in vivo study to use a panoramic approach to evaluate changes in every immunologic component and identify the clinical response due to LEN treatment by applying correlative analysis method.

Human regulatory T cells (T-regs) are defined as $CD4^+CD25^{bright}CD127^{dim}FOXP3^+$ subset of T cells whose potent immunosuppressive capacity protects the host from auto-reactivity and maintains immune homeostasis during infection and after transplantation.[21] In our study results, we unexpectedly found that LEN increased the numbers of T-regs in the peripheral blood in majority of the participating patients. Our observance on T-regs was unforeseen for LEN reportedly inhibits proliferation and function of T-reg cells.[22] Moreover, clinically, elevated intra-tumoral T-reg frequency is correlated with poor prognosis and reduced survival rate in various types of cancer settings.[23]

Although it is commonly assumed that elevated T-regs correlate with a negative clinical outcome in a variety of solid tumors, the role of T-regs in KS and their response to LEN have not been studied prior to our study. For patients available for our correlative study, significant increase in peripheral T-reg number

as well as infiltrating T-regs in the microenvironment of the tumor correlated with

positive clinical response (decrease in tumor burden). This correlative data

enable us to speculate on three novel concepts: 1) LEN increases the frequency

of circulating T-reg-phenotype cells, 2) LEN's anti-angiogenic mechanism may

be due to T-reg induction, and 3) In contrast to their role in promoting tumor

growth by inhibiting tumor immunosurveillance, T-regs may inhibit

angioproliferative tumors such as KS.

MTD of LEN in AIDS-KS patients was determined from phase I study (N=16) and 15 additional patients enrolled in phase II at MTD.

16 patients with AIDS-KS participated in phase I LEN study. Each cohort in this dose escalated study consisted of 3 patients in the order of enrollment. Patient identification number1-3, 4-6, and 7-9, received 10 mg/day, 15 mg/day, 20 mg/day respectively. Patient 10 – 16 received the maximum dose of 25 mg/day. (Figure 1A) Commonly reported adverse events were fatigue, neutropenia, and diarrhea, which were predicted for similar symptoms were previously reported related to LEN. Rare adverse events consisted of lung infection, fever, dizziness, and facial numbness. We were unable to obtain the clinical reports on all of the patients because AMC did not disclose information on some of recent participants as phase II is still an on-going study. However, significant number of patients tolerated LEN without any major complications and the trial was admitted into phase II with the maximum tolerated dose (MTD) of 25 mg/day.

15 additional patients (#17 – 31) were enrolled at 25 mg/day as phase II participants. One cycle consists of 21 days of daily LEN administration then a 7-day break as illustrated in figure 1B. Although the complete clinical response in all enrolled patients is yet to be concluded, overall, 12 patients (38.7%) continued the study past cycle 6 and 3 patients (9.7%) completed all 12 cycles.

Both scenarios indicate partial response (PR) achievements at minimum according to the predefined PR criteria. 6 patients (19.4%) discontinued after 6 cycles due to reasons that are not disclosed though our surmise is that the cause of discontinuation was most likely due to stable disease rather than intolerable side effects. Figure 1C shows the duration of LEN administration for the patients who either completed or withdrew from the study. Aside from MTD, there was no evidence of dosage dependence in terms of the patient's clinical response to LEN.

Gating strategy for phenotypic analysis of lymphocyte subsets.

Whole blood samples from all the patients were evaluated for broad parameters of the leukocytes using flow cytometry. The leukocyte subsets of interest were as listed: total lymphocytes, total T, CD4$^+$ T ($\alpha\beta$ and $\gamma\delta$), CD8+ T ($\alpha\beta$ and $\gamma\delta$), NK, NKG2D, monocytes, granulocytes, B cells, and T-regs. (Figure 2A and 2B) Other parameters such as programmed death (PD)1 expression on T-reg, NK-T, and others were not shown. Figure 2 illustrates representative gating strategies for each of leukocyte subsets according to their known phenotypic surface marker. Although CD 25 is known to be the prominent marker for T-reg identification, the ability to differentiate between T activated (T-act) cells and T-reg is limited since CD25 expression is up-regulated in T cells during early activation.[25] In order to determine if CD4$^+$CD69^{dim}CD25^{bright} phenotype was true representation of T-regs, we performed concomitant phenotypic staining with

another informative surface marker for T -regs, CD127 (the α chain of IL-7), as well as intracellular staining with FOXP3, transcription factor forkhead box P3 specific for T-reg lineage. (Figure 2B) Homogenous FOXP3 expression was unique in CD4$^+$CD25^{bright}CD127^{dim}, not in other populations including CD4$^+$CD25^{dim}CD127$^{dim/bright}$ cells. FOXP3 expression in non-T-reg population data is not shown.

Peripheral T-regs were increased in AIDS-KS patients during the LEN treatment.

We examined the effect of LEN on various blood leukocyte subsets over the period of the treatment. We did not observe remarkable responses in NK, NK-T, monocyte, granulocyte, B cell, CD4$^+$ T, or CD8$^+$ T cell numbers (data not shown). Interestingly, of the 26 evaluable patients with baseline T-reg data, 23 (88.5%) of patients showed increase in peripheral T-reg frequency. Most robust response in each patient was observed in the early stage of the LEN treatment, which was between baseline at day 0 and day 84. (Figure 3A) Since we obtained most data from earlier time points (before treatment discontinuation of many of those patients), we focused on T-reg frequency at three time points in the early stage of the study: baseline (pre-LEN), day 6 (post-LEN), and day 13 (post-LEN). (Figure 3B) Compared to the baseline value (average T-reg %= 5.3%), percentages at day 6 (average = 7.4%) and day 13 (average=12.1%) were significantly elevated. Figure 3C shows increase of T-reg frequency from baseline to peak value observed throughout the study. Degree of T-reg amplification

ranged from 4.6% in patient 25 to 24.1% in patient 7, and the average frequency

increase among the T-reg responders was 10.44%. Horizontal line (Y=5%)

represents reference T-reg percentage of CD4^{+} T cells observed in healthy adults.

[26] In the peripheral blood of HIV infected adults under HAART management, but

in absence of tumor, similar value of 5% T-reg frequency was detected, which

confirms the validity of the reference range used in this analysis.[27]

Extensive study of four UCSD patients showed inverse correlation between T- reg
response and clinical response measured by tumor burden.

Extensive monitoring of peripheral immunologic response and tumor

burden was performed on four patients (patient ID# 5,7,11, and 12) enrolled in

the same study site. (Figure 4A; only T, CD4^{+}, CD8^{+}, and T-reg data shown) In

parallel to our findings from the comprehensive analysis of all patients, T-regs

were increased in all four of the patients. Two of the patients (7 and 12)

achieved higher CD4^{+}CD25^{+}CD69^{-} T cell peak percentages than the other two (5

and 11). For patient 12, the baseline CD4^{+}CD25^{+} CD69^{-} T cell number was

5.19x10/mm^{3} (10.5% of CD4^{+} T cells) compared to 20.9x10/mm^{3} (29.4% of CD4^{+} T

cells) at peak. Comparatively, patient 11 had a mild increase from the baseline

count at 0.41 x10/ mm[3] (1.1% of CD4^{+} T cells) to 3.5 x10/ mm[3] (7.2 % of CD4^{+} T

cells) at peak. (Figure 4B)

To study clinical response of LEN administration, we monitored each

patient's tumor burden by summing the cutaneous lesion area from five different

representative body sites. By the end of the treatment, we observed that the total lesion area for three patients (patient 5, 7, and 12) decreased (8.3-44.8%), whereas for patient 11, the lesion area increased by 42.5% (Figure 5A). Interestingly, all patients displayed an initial decrease in lesion size, but for patients 5 and 11, the lesions subsequently increased following an upward trajectory curve. We also monitored tumor counts by their characteristics, raised or flat. Similar to T-reg and the lesion size response, patient 5 and 11 had an increase in total number of tumor lesions in the representative area, 20% and 1.8%, respectively, whereas patient 7 and 12 showed reduction by 55.4% and 30.8%. In terms of their morphological changes in cutaneous lesions, patient 5 and 12 demonstrated significant flattening of the nodules, though total count for patient 5 increased by the end of the study. Given that patient 5 and 11 discontinued their therapy, we defined them as non-responders, whereas patients 7 and 12 are responders based on the large decrease in lesion size and total count upon LEN treatment as well as their completion of the complete regimen.

FOXP3+ T-regs are recruited into the KS tumor microenvironment.

In order to rule out the possibility that decline in T-reg percentage is attributed to T activated cells being recruited to the tumor microenvironment thus exiting the periphery, we monitored changes in FOXP3 expression in KS lesion taken at pre-treatment (baseline) and post-treatment (day 15). We postulated

that day 15 would be a good time point for observing a robust immune response caused by the drug. Figure 6A represents immunohistochemistry performed on KS biopsies from patient 12; FOXP3 expressing cells are shown as red-brown stain. Compared to the lesion from pre-treatment, post-treatment lesion illustrated increased amount of dark brown staining. Quantitation of infiltrating T-regs was analyzed by counting the FOXP3$^+$ stain in three representative areas of the lesion that had the most positive cells observed under 20 x magnifications. Mirroring results seen in tumor burden assessment, LEN induced a significant accumulation of FOXP3 expressing T-regs in the KS lesion of the responders relative to the non-responders.(Figure 6B) This further strengthens the hypothesis that T-reg may have a beneficial role in suppressing hyperproliferation of malignant cells, possibly by playing a crucial part within the microenvironment of the tumor.

All sections in full are an adapted version of materials that is currently being prepared for submission for publication. Sumit Arora, Erin Reid, Kelly Shimabukuro, Sepi Mahooti, Chanae Hardamon ; Bui, Jack. The thesis author was the primary author of this material.
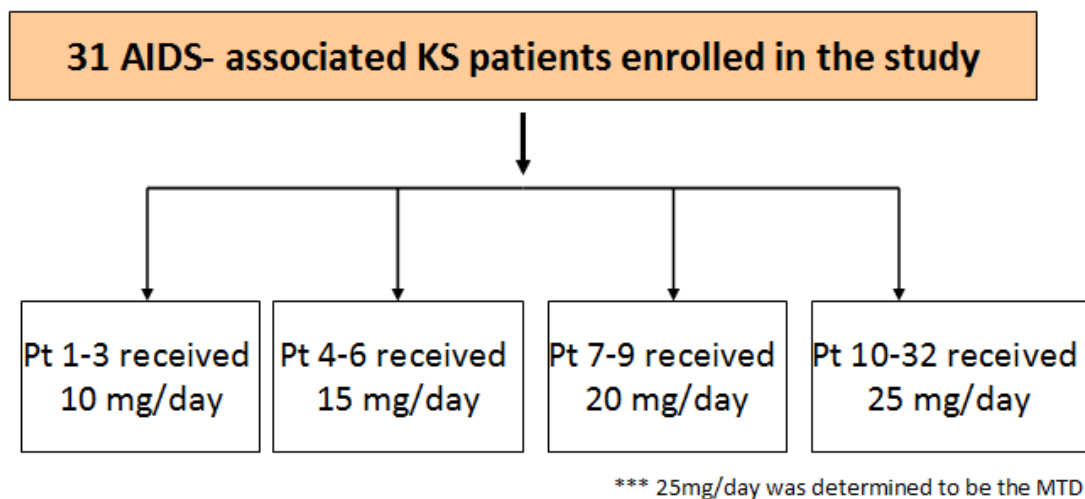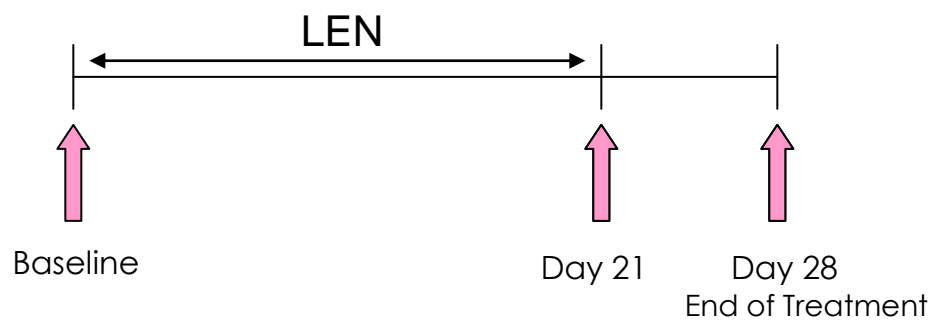
A.

31 AIDS- associated KS patients enrolled in the study

| Pt 1-3 received 10 mg/day | Pt 4-6 received 15 mg/day | Pt 7-9 received 20 mg/day | Pt 10-32 received 25 mg/day |

*** 25mg/day was determined to be the MTD

Figure 1: Study design and duration of LEN of patients enrolled in phase I/II study.
(A) Total 31 patients with AIDS-KS were enrolled in the open label, multicenter, phase I/II study of LEN as a single agent. Study design for phase I utilized the standard 3+3 design for escalation of dose to form four different cohorts. First three patients (1-3) received the lowest dose of 10 mg/day, subsequent groups of three received escalated dose in 5 mg increments. Phase I included patients 1-16. Phase II included 15 additional patients (17-31) who continued on 25mg/day dose treatment plan. 25mg of LEN was determined to be the maximum tolerable dosage because AMC concluded that the majority of patients from phase I trial tolerated the dosage without developing extensive adverse effect.
(B) LEN was administered orally on 21 days of a 28-day cycle.
(C)Total of 24 patients have participated in the phase I/II study of LEN. Other 7 patients, whose data are not shown, are currently enrolled in phase II. 3 patients (7,12,and 13) completed 12 cycles (day 334) of LEN treatment, 5 patients (14,15,22,24, and 26) continued the treatment beyond cycle 6 (day 166), 6 patients (1,2,3,9,18, and 21) finished 6 cycles, and 10 patients discontinued the treatment before reaching cycle 6. Reasons for withdrawal were not disclosed by the clinical sites.

B.

LEN

Baseline   Day 21   Day 28
End of Treatment

Figure 1: Continued.

C.

Days on LEN before treatment discontinuation

Figure 1: Continued.

A.

B.

Figure 2: Representative flow cytometry plots demonstrating gating strategies.
(A) Representative flow cytometry plots demonstrate the gating strategies used to identify cell populations depending on their phenotypic markers. We initially gated on live cells= 7AAD&FSC and lymphocytes= SSC&FSC. From the live gate, granulocytes (SSC & CD45), and monocytes (SSC & CD4) were identified. From the lymphocyte gate subset of lymphocytes were defined: NK cell (CD3$^-$CD56$^+$), NKG2D expressing cell (CD56$^+$NKG2D$^+$), CD4 cell (CD3$^+$CD4$^+$CD8$^-$), CD4$\alpha\beta$ & $\gamma\delta$ CD8 cell (CD3$^+$CD4$^-$CD8$^+$), CD8 $\alpha\beta$ & $\gamma\delta$, B cell (CD3$^-$CD20$^+$), and T regulatory cell (CD4$^+$CD56$^+$CD69$^-$).
(B) Representative flow cytometry plots show two different gating strategies for T-reg-phenotype cells in PBMCs from patient 12. Intracellular expression of FOXP3 was obtained from the same patient sample. Intracellular FOXP3$^+$ population was gated from CD4$^+$CD25$^+$CD127$^-$ population.
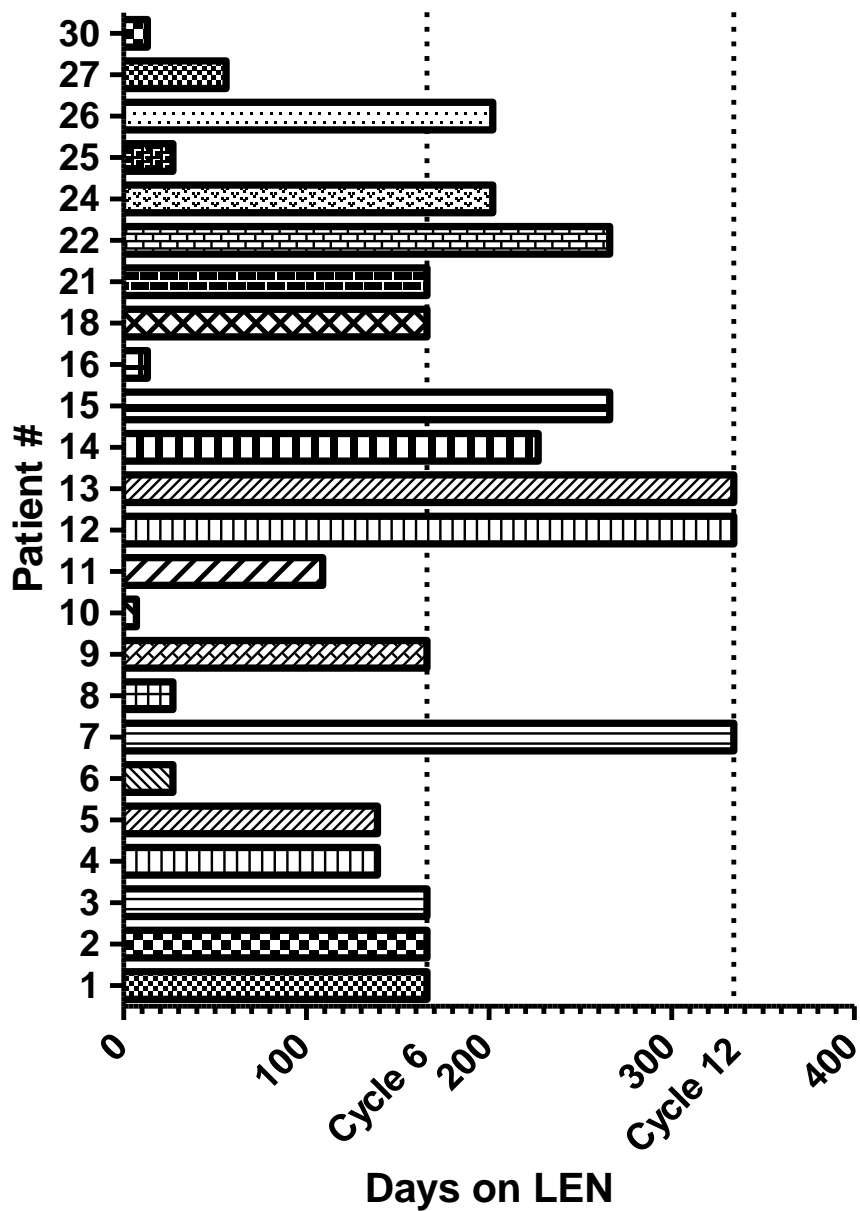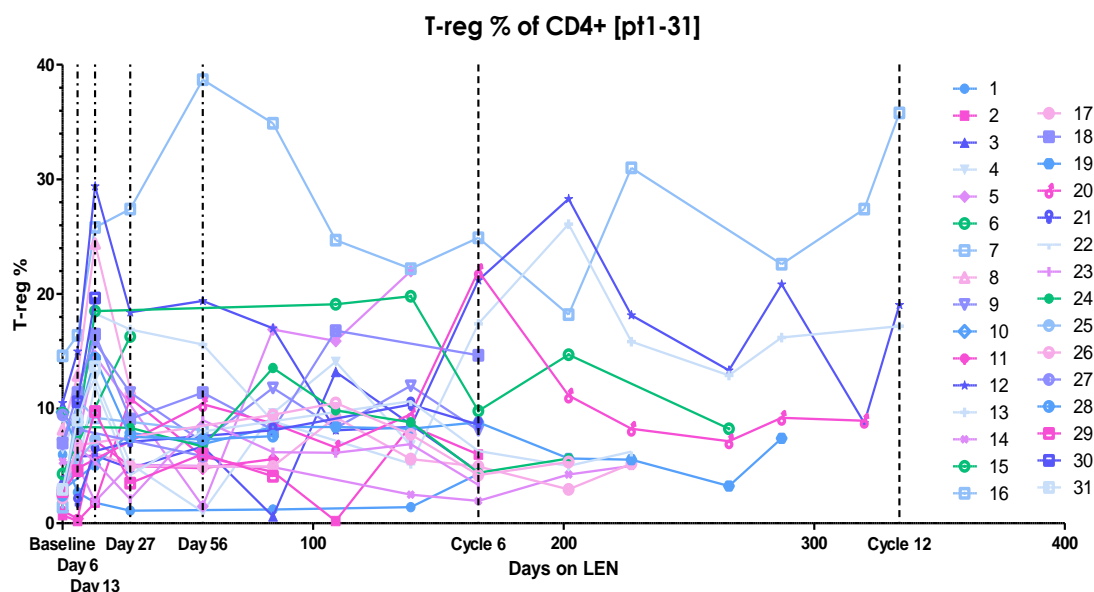
A.

Figure 3: T-regulatory response observed in all 31 patients enrolled in the study.
(A) A trend for T-regulatory percentage of CD4+ T cells through out LEN treatment for each of 31 patients is plotted. Vertical dotted lines represent blood collection dates: baseline (day0), day 6, 13, 27, 56, 166 (cycle6), and 334 (cycle12; study completion). Not all collection dates were indicated with a vertical line.
(B) T-reg percentage at three different time points: day0, 6, and 13. T test values for statistical significance were <0.01 and <0.001, from paired one tail distribution T test, from comparing all the values from baseline to day 6 or to day 13 respectively.
(C) Patients receiving different dose is grouped together to assess dose dependency of T-reg response. A bar represents the different between baseline T-reg value and the peak value during the duration of the LEN study. Horizontal dotted line (Y=5) represents the reference T-reg percentage of CD4+ cells in the peripheral blood of healthy adults.
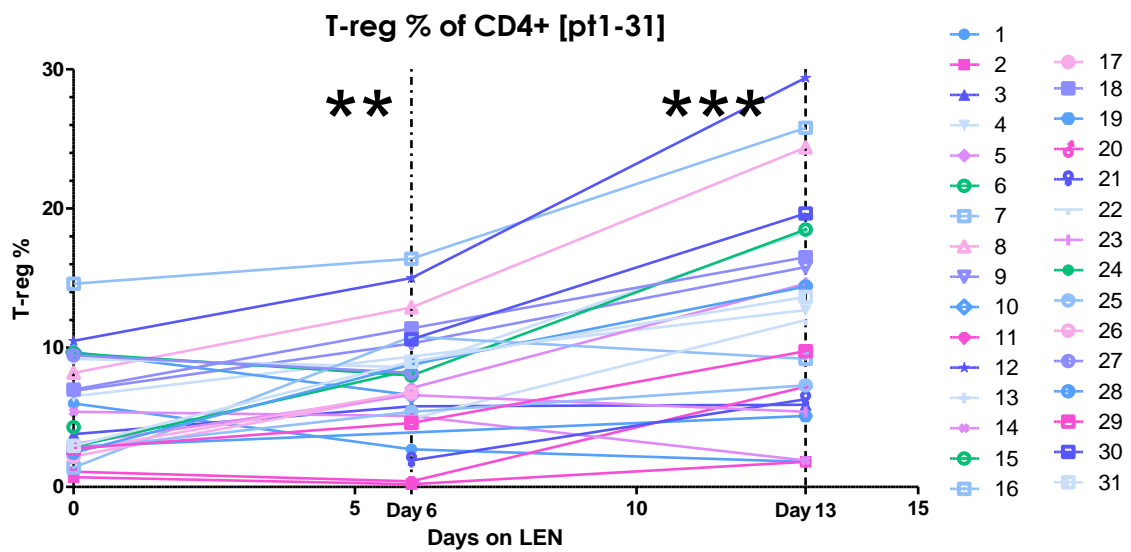
B.

Figure 3: Continued
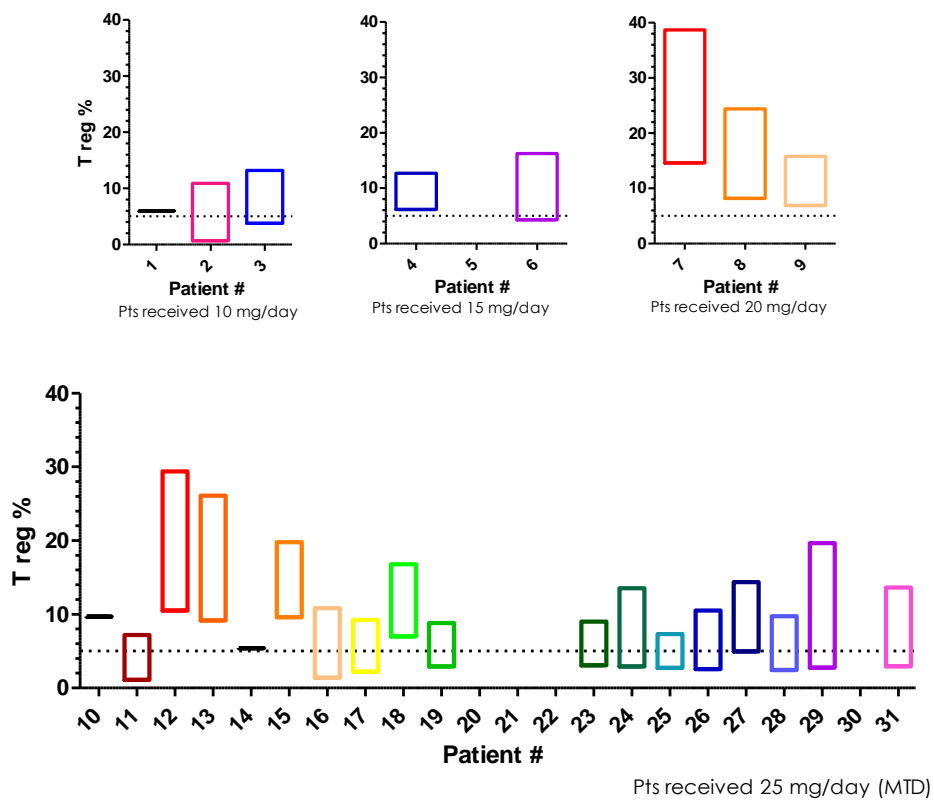
C.

Treg % Baseline-Peak
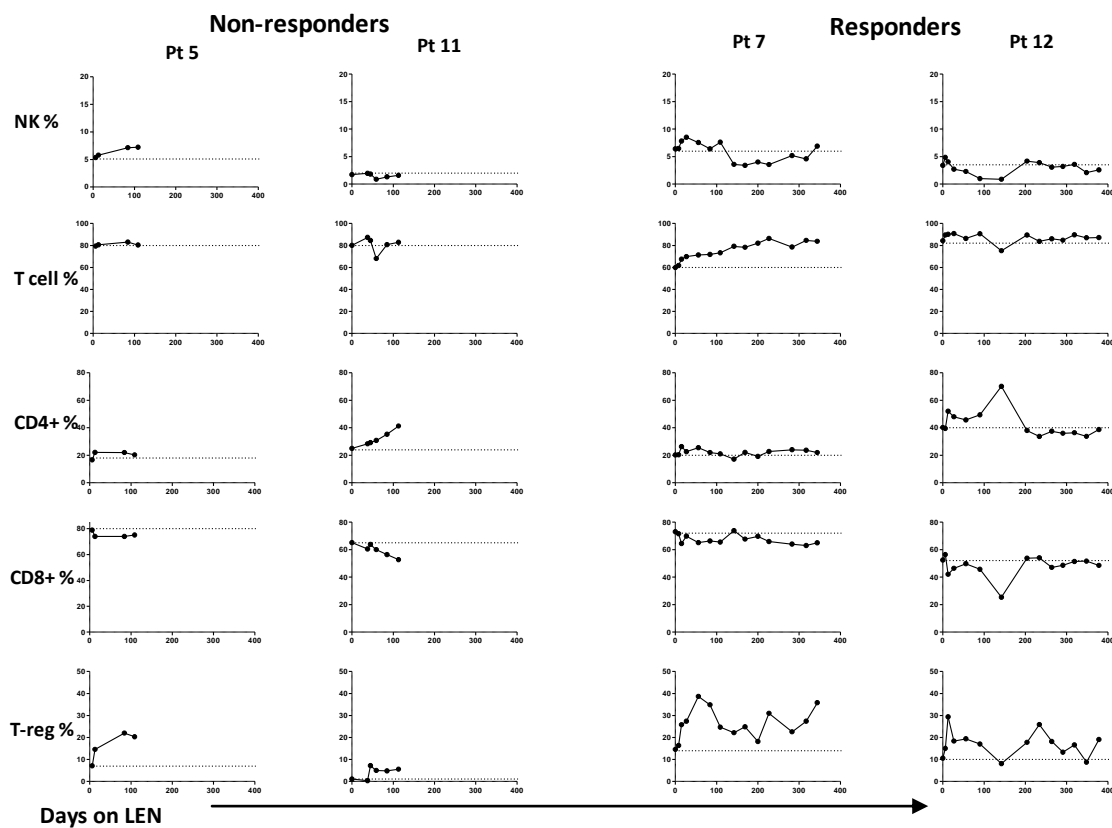

Figure 3: Continued

A.

Figure 4: Monitoring the frequency (%) and absolute count (x10/mm³) of subtype of immune cells in the PBMC of selected patients (n=4).

(A) Frequency values of NK, T, CD4⁺,CD8⁺, and T-reg of CD4⁺ was followed from pre-treatment (day 0) to end of the study. Non-responders (patient 5 and 11) were not able to finish the study whereas responders (patients 7 and 12) completed all 12 cycles. Dotted horizontal line represents their initial value at baseline. Subsequent points from different time points of the study demonstrate deviations from the baseline.
(B) Absolute count of lymphocytes, CD8+ T, CD4+ T, and T-regs were calculated from the complete blood count report provided from clinical site where all four patients enrolled. Lymphocyte counts from time points other than the designated visit dates were also included to show a more detailed trend.
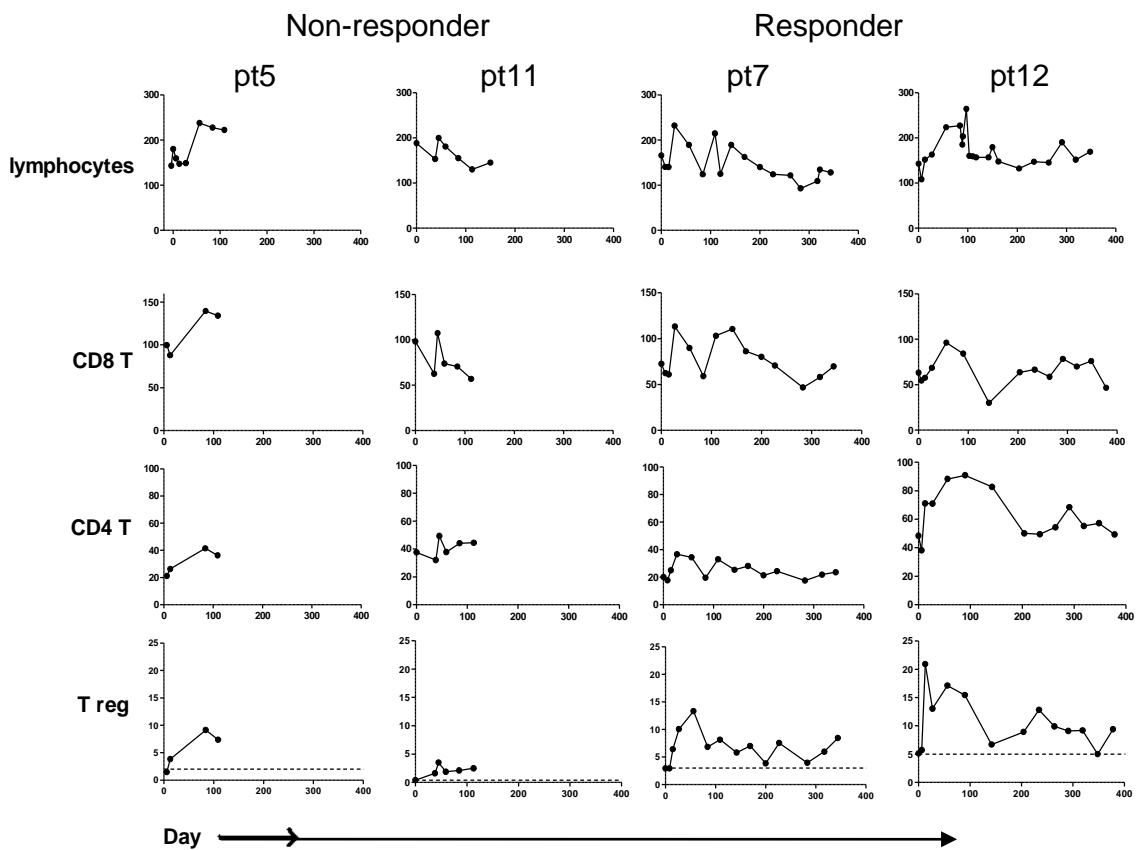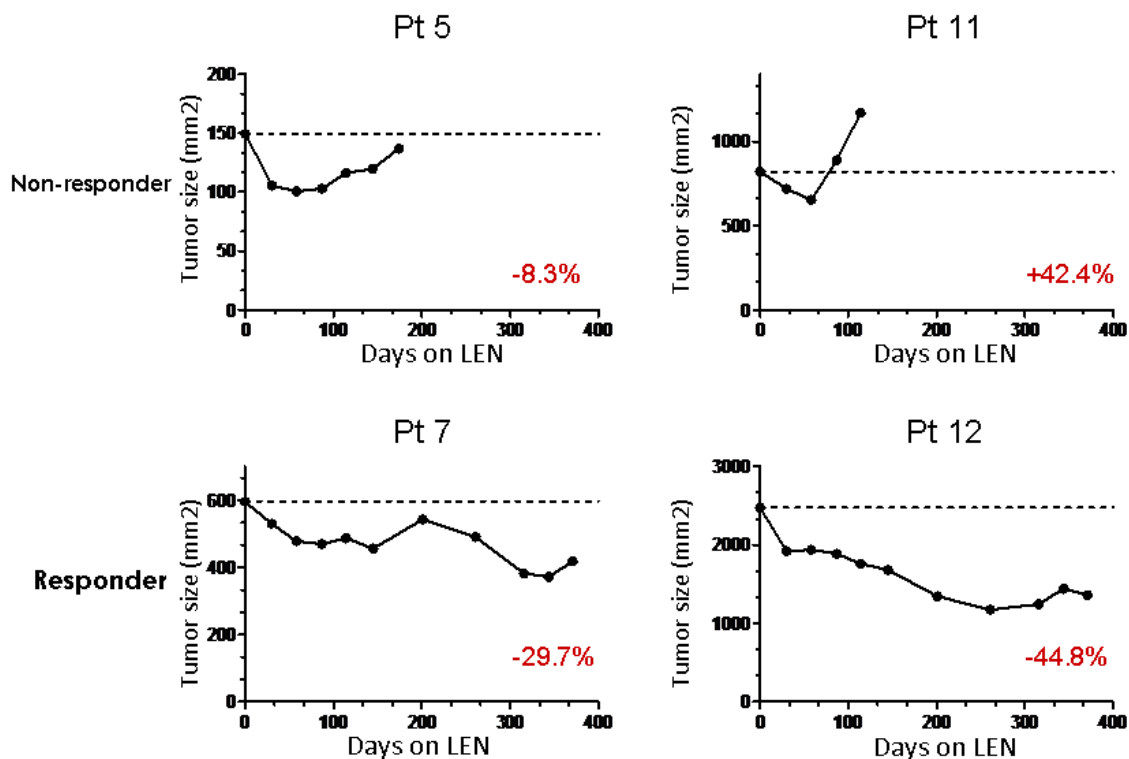
B.

Figure 4: Continued

A.

Figure 5: Tumor burden assessment in the selected patients (n=4) show reduction in lesion size (mm²) and number during LEN treatment.
(A) Sum of five representative cutaneous lesion sizes (length x width) was monitored by following the same lesion chosen at the baseline. In order to avoid any discrepancies, same physician measured the lesions at each visit.
(B) Number of flat and raised lesions in three representative areas on the body was added to give total number of lesions. Percent change shown in both figures A and B was calculated using values from baseline and end of the treatment. The Y axis was not normalized due to range variations between the patients.
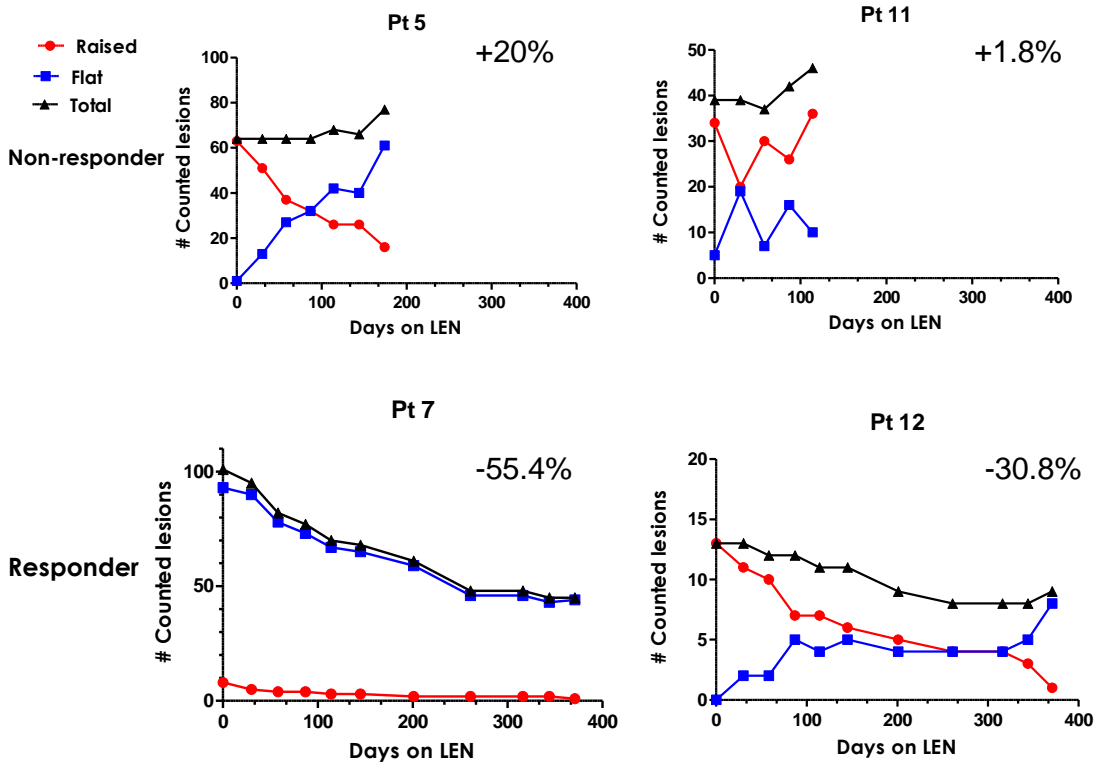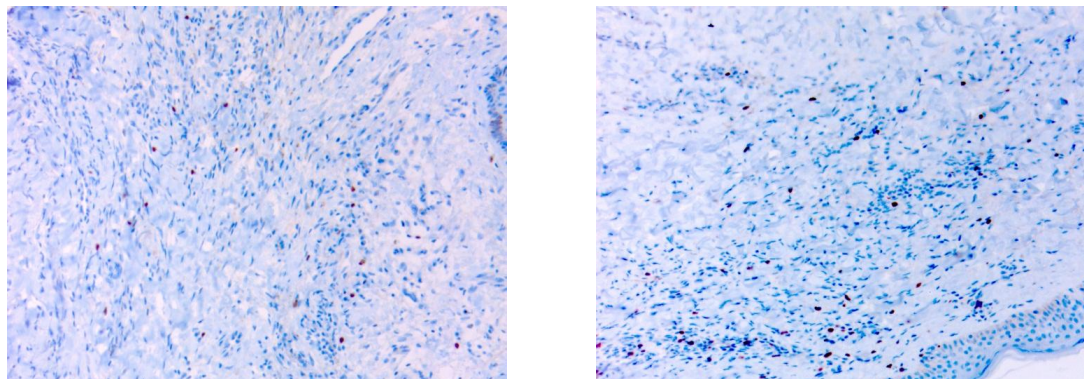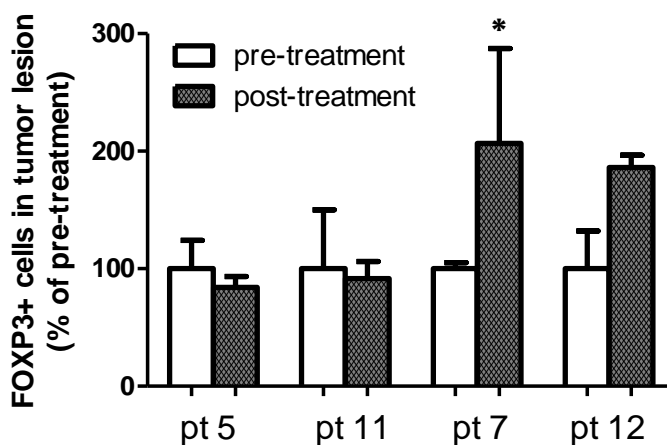
B.

Figure 5: Continued

A.

Pt 12 pre-treatment (D0)   Pt 12 post-treatment (D15)

B.

Figure 6: FOXP3 immunohistochemistry of KS lesion.
(A) Histopathological photomicrograph illustrates FOXP3 expression in the dermis of a cutaneous KS lesion from patient 12 (20x). 3mm section from the patient's KS lesion biopsy was stained with FOXP3 antibody. FOXP3+ cells were quantified at two different time points: pretreatment (baseline) and post-treatment (day 15). The biopsies were obtained from separate representative lesions on separate days to exclude biopsy-induced changes.
(B) Patients 7 and 12 had increased FOXP3 staining at day 15 compared to the baseline (*statistical significance: P value <0.05). Average of three representative areas on day 0 was set as the reference value and was compared to the average value on day 15.

DISCUSSION

Our results obtained from various approaches converge to suggest LEN as a potent inducer of anti-tumor activity. Our speculation is based on the finding that unusual amplification of circulating T-regs was observed in KS patients following the LEN treatment. (Although current literature demonstrates a direct relationship between chronic HIV infection and elevated frequency of T-regs, we would like to note that our findings regarding T-regs are independent of the patient's infection status as HAART regimen is documented to normalize the frequency of circulating T-regs.[27])Furthermore, such T-reg response correlated to a positive clinical response in the patients, which was determined by monitoring the size and number of their cutaneous KS lesions. Consistent with peripheral T-reg response, we observed increased infiltration of T-regs in the KS lesions of the responding patients, which strengthened our proposition that T-reg may induce an anti-angiogenic activity within the tumor. Such infiltrating T-reg response observed only 15 days after the initiation of LEN treatment demonstrates that LEN could potentially serve as a biomarker indicating selection of therapy. Absense of early increase of T-regs in the KS biopsy section would be an effective indicator to predict the cost and time efficient endpoint of the treatment.

In attempts to explain the mode of T-reg increase, two critical questions loom: is the percentage increase due to T-reg proliferation or from death or escaping (to the tumor site) of other lymphocyte subsets initially residing in the peripheral blood. One suggestion for the increase in absolute count of T-reg

could be attributed to rise in total T lymphocyte since T-reg is a subset

oflymphocytes. However, the change in T-reg percentage of CD4 [+] population

did not reflect any changes in the total T cell percentage of total lymphocytes.

Thus we justify the T-reg expansion in the blood is not necessarily from the

increase in the lymphocytes as a whole. The accrual of intra-tumoral T-regs also

rules out the possibility that T-reg frequency in blood is increased in response to T-

acts being recruited out to the tissues involving KS. We hypothesize that the

increase of circulating T-reg percentage is rather due to either cell proliferation

induced by cytokines or enhanced differentiation from naïve T cells rather.

Functional T-regs are generated from naïve T-reg cells with the help of CD28 and

IL-2[28] and increased IL-2 due to LEN could possibly drive the rise in FOXP3^{+} T-regs.

Nevertheless, solidifying such reasoning needs further investigation with cytokine

studies.

    In contrast to the popular tendency to believe that T-reg level and

disease progression are inversely correlated, we would like to highlight in vivo

studies where T-regs partake in anti-tumorigenic activity in certain types of tumors

and suggest few possible mechanisms of action. We propose that one of the

unveiled functions of T-reg may be anti-tumorigenicity in a sarcoma model

contrary to the popular belief that T-regs play a favorable role in tumor growth

by maintaining immunological quiescence. Number of reports suggest

localization of intratumoral FOXP3^{+} T-regs infiltration is associated with favorable

prognosis for certain types of malignancies including colorectal carcinoma,

head and neck carcinoma, B cell lymphoma, and Hodgkin's lymphoma [29],

which coincide with our findings. One possible mode of mechanism of T-reg as an anti-tumor agent is through reprogramming of pre-existing mature T-regs into a helper T cell-like phenotype. An in vivo study in mice showed that cells of FOXP3^{+} lineage can be converted to perform cross-presentation for priming of CD 8^{+} T cells via expression of CD40 L. Surprisingly, immune deficient mice receiving exogenous reprogrammed T-reg cell fraction showed a robust proliferative response to vaccination. [30] Differentiation and subsequent proliferation of reprogrammed T-regs in the AIDS-KS patients receiving LEN could explain the role of T-regs in supplementing the fight against tumor. However, whether such phenomenon can be regenerated in humans, more so in AIDS-KS setting, is the crucial question that remains to be investigated.

HHV-8 produced LANA protein pirates normal cellular processes that creating the appropriate environment for tumor proliferation. A specific example is down-regulation of TβRII on the surface of infected endothelial cells by histone modification and DNA methylation at the promoter site that will repress the receptor expression. [8] TGF-β has anti-proliferative and apoptotic effects on epithelial, endothelial, and hematopoietic cells. [31] Deficiency in TGF-β leads to hyperproliferation in effective cells and cause tumor progression, which is the consequence similar to loss of TGF-β sensitivity. Down-regulation of TβIIR would lessen the responsiveness to the exogenous signaling to limit the cell growth. It is possible that LEN is inducing TGF-β production of T-regs and elevated production of TGF-β consequently compensates for limited responsiveness in KS cells with less TβRII expression. Overcoming effect would sensitize KS cells to apoptotic and

cytostatic effects of TGF-β signaling, ultimately opposing angiogenesis. These suggestive mechanism might help explain, in part, the counter-intuitive role of T-regs in KS. In order to assess the response in KS to TGF- β, we plan to conduct an in vitro experiment with a KS cell line and monitor the cytolytic activity of LEN treated KS cells.

CONCLUSION

In conclusion, LEN therapy for AIDS-KS patients induced significant increases in the percentage of phenotypic T-regs in the peripheral blood in majority of patients examined. Comparison between responders and non-responders indicated that the increase in phenotypic T-reg levels in the blood and an early increase in FOXP3 staining at the tumor site correlated with long-term responsiveness to LEN therapy. These results are paradoxic and unexpected, since T-regs have well-known tumor-promoting properties in part because of their immune suppressive function.[16] We speculate that T-regs may have anti-angiogenic activity and may specifically inhibit angioproliferative tumors. Indeed, they are known to express TGF-β, which can induce apoptosis in endothelial cells. As a future study in vitro cell apoptosis assay by treating KS cells with TGF-β will be performed to test our hypothesis. Another planned study is to examine the RNA expression from the KS lesions of the patients at two time points, baseline and day 15. Monitoring the mRNA expression at two different time points will allow us to characterize which KSHV mRNAs are present in patients prior to therapy with LEN and which cellular mRNAs are altered by LEN in KS. Furthermore, comparing between responders and non-responders will enable us to determine if the mRNA profiles correlated with clinical response.

Our results call attention to the dearth of literature on the effects of LEN in human patients and highlight the possibility that the clinical effects of LEN may

be mediated by its stimulation of T-regs. Further studies are required to address LEN's place in the cancer field, however, our preliminary results suggest LEN's potential to be part of the therapeutic arsenal used in combating AIDS-KS, especially in patients who respond by induction of T-reg-phenotype cells.

MATERIALS AND METHODS

Study design

This phase I/II multicenter clinical trial of oral Lenalidomide (LEN) in patients with AIDS- associated Kaposi's sarcoma (AIDS-KS) was an open label, single agent, dose escalation study with expansion of cohort in phase II at maximum tolerated dose (MTD) determined in phase I. LEN was provided by the manufacturer (Celgene). All 31 AIDS-KS patients enrolled in the study provided informed consent according to the institutional guidelines approved by National Cancer Institute (NCI) Institutional Review Boards (IRB). HIV sero-positive patients who were at least 18 years of age with biopsy-proven KS were eligible for the study. Inclusion criteria required for measureable cutaneous lesions amenable to four 3-mm tumor biopsies and CD 4 count > 50/mm^3 and HIV viral load < 2,000 copies/ mL. Patients must be on a stable antiretroviral therapy regimen for HIV infection for greater than 12 weeks prior to the time of study entry. All participating sites were registered with the AIDS malignancy consortium Operations and Data Management Center (AMC ODMC).

Treatment plan and response criteria

In phase I, the Len dose will be given to four different cohorts of patients at the predetermined dose levels: 10 mg/day, 15 mg/day, 20 mg/day, and 25

mg/day. Dose escalation is performed using the 3+3 design where the first cohort with three patients received the lowest dose and subsequent cohort received the next higher dose if observed toxicity is <33%.

In phase II, additional patients enrolled in the study at the MTD determined in phase I.

Patients were given daily dosage of LEN for 21 days of a 28-day cycle. Up to 12 cycles (48 weeks) was allowed provided that the patient had a partial or complete response. LEN treatment was discontinued if the patient experienced tumor progression, intolerable adverse effects related to LEN, or stable disease by cycles 6 (24 weeks). Complete response (CR) is defined as the absence of any detectable residual KS whereas partial response (PR) is defined as disease containment without new lesions and ≥50% reduction in the number of lesions or in the sum of the products of the largest perpendicular diameters of the marker lesions. Progressive disease (PD) is defined as $\geq 25\%$ increase in the sum of the perpendicular diameters, $\geq 25\%$ increase in total number of lesions, or addition of five new lesions on the body. Stable disease pertains to none of the above. Stable disease was referred to a disease state that did not meet any of the above criteria.

Biologic endpoints for sample collection

Peripheral blood was collected at day 1, 8, 15, and day 1 of subsequent cycles, and at the time of treatment discontinuation. Peripheral blood samples

were used for flow cytometric analysis and various in vitro studies. Two 3 mm KS tumor biopsies from a KS lesion were prepared at day 1 and day 15. Biopsy at later time point was not obtained. One biopsy specimen was stored in RNAlater for the purpose of KSHV/HHV-8 transcription profiling and the other specimen was stored in formalin for immunohistochemistry at a later time.

Tumor assessment

Tumor burden was evaluated by measuring the size of the selected skin lesions at day 1 of each cycle up to cycle 6. Subsequent measurements were recorded at day 1 of every other cycle. In order to avoid variability, the same area or lesion was measured to track changes once a representative area or lesion was chosen. To monitor changes in lesion size, five marker lesions of largest size with clearly defined margins were chosen. For evaluating changes in number and characteristics of the lesions, three representative areas were selected. Within the area numbers of flat and raised lesions were documented separately. These selected lesions were representative of other lesions on the body and similar in characteristics in nodularity, size, and color.

Multiparameter Flow Cytometric Analysis.

Fresh blood samples were transported to Bui lab (University of California, San Diego) in EDTA tubes on the day of collection. From the whole blood

peripheral blood mononuclear cells (PBMC) were separated by centrifugation at 2500 xg for 20 minutes then isolated after removing of red blood cell with the lysis buffer (Sigma). After washing with stain buffer (90% 1x PBS, 1% Fetal Bovine Serum (Atlanta Biologicals), and 1% sodium azide), cells were stained with fluorochrome conjugated antibodies stained with antibodies specific for the following antigens: CD3, CD4, CD8, CD16, NKG2D, DR, CD56, CD25, CD69, CD127, $\alpha\beta$ TCR, $\gamma\delta$ TCR, CD20, B7H1, CD206, and Va24Ja18 (eBioscience). Minimum incubation period was 15 minutes in the dark at 4°C. Then the cells were washed off of the excess antibodies and 7-aminoactinomycin D (7AAD) in DMSO (Sigma) was added at 1µg/ mL. The cells were fixed with 2% formaldehyde in PBS (Cellgro).

For intracellular cytokine stainings, cells were initially stained with surface antibodies. FOXP3 intracellular staining kit from eBioscience was used. After fixation and permeabilization with perm and fix buffer, cells were stained with intracellular antibodies (anti-FOXP3 or IgG 1 k isotype (BD Biosciences)) in 100uL of perm buffer over night. Samples were acquired on a flow cytometric device using the FACS DiVa software (BD Biosciences). Data analysis was performed with the FlowJo software package (TreeStar), Microsoft Excel, and Graph Pad Prism 5.

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