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Serial assessment of lymphocytes and apoptosis in the prostate during coordinated intraprostatic dendritic cell injection and radiotherapy

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Abstract

Local radiotherapy plus intratumoral syngeneic dendritic cell injection can mediate apoptosis/cell death and immunological tumor eradication in murine models. A novel method of coordinated intraprostatic, autologous dendritic cell injection together with radiation therapy was prospectively evaluated in five HLA-A2⁺ subjects with high-risk, localized prostate cancer, using androgen suppression, 45 Gy external beam radiation therapy in 25 fractions over 5 weeks, dendritic cell injections after fractions 5, 15 and 25 and then interstitial radioactive seed placement. Serial prostate biopsies before and during treatment showed increased apoptotic cells and parenchymal distribution of CD8⁺ cells. CD8⁺ T-cell responses to test peptides were assessed using an enzyme-linked immunosorbent spot IFN- γ production assay, demonstrating some prostate cancer-specific protein-derived peptides associated with increased titer. In conclusion, the technique was feasible and well-tolerated and specific immune responses were observable. Future trials could further test the utility of this approach and improve on temporal coordination of intratumoral dendritic cell introduction with particular timelines of therapy-induced apoptosis.

Keywords

immunotherapy; prostate; radiation; therapy

Prostate cancer is the most common noncutaneous malignancy in the USA, with an estimated 241,740 new cases and 28,170 deaths in 2011 [101]. The problem of late biochemical recurrence after definitive therapy likely represents the outcome from

micrometastatic disease at the time of definitive therapy. The proportion of men who have later biochemical failure can be predicted by pretreatment disease features, including prostate-specific antigen (PSA) level, Gleason score and T stage, as well as treatment details.

The addition of hormonal therapy to local radiation therapy has been studied by the Radiation Therapy Oncology Group (RTOG) and shown to improve progression-free and overall survival in some subsets, particularly those with higher recurrence risk [1–3]. However, the recurrence risk persists and adverse effects of escalation of local treatment may be significant. For this reason, new approaches that improve both cure frequency and treatment tolerability are needed. The addition of immunotherapy to standard therapy is attractive for several theoretical reasons including simultaneous treatment of local and distant (microscopic) residual disease and the possible induction of ‘memory’ immunity preventing late recurrences. Indeed, clinically localized, high recurrence risk prostate cancer presents some ideal features for development as a target for novel immunological treatment as:

- The macroscopic disease has been treated;
- The anticipated time to recurrence may be long enough to develop a useful immune response;
- The metastatic burden is microscopic and small in total bulk;
- The corresponding tumor effect on the general immunophenotype should be minimal, which is in contrast to general immune activating approaches.

The particular details of the antigen-presentation process in patients during radiation therapy are not well characterized [4]. The immunogenicity of the epitopes of tumor-specific antigens and the capacity of the tumor to modulate the phenotype of the APCs to generally favor tolerance are believed to be key elements determining the capacity for acquisition of anticancer immunity [5,6]. Numerous factors present in cancer patients severely limit the ability to mount host antitumor immune responses. Among these factors are Tregs, myeloid-derived suppressor cells, defective dendritic cells (DCs) and various immune-suppressive cytokines [7,8]. It is therefore clear that the combination of different therapeutic modalities offers the opportunity to provide desirable clinical results [9].

Local tumor irradiation could be one of such modalities. It can disrupt tumor stroma, thus allowing T cells to penetrate tumor parenchyma, limit the negative effect of immune suppressive factors, as well as to release large number of antigens associated with apoptotic tumor cells, an approach tested previously by Nikitina and Gabilovich [10]. Different fractionation methods could be differentially immunogenic. In murine models, intratumoral injection of syngeneic DCs were ineffective alone, but promoted acquisition of curative immunity when given coordinated with radiation therapy, even with the radiation therapy at a dose that is not curative. In tumor-bearing mice, those treated with external beam radiation therapy (EBRT) and intratumoral DC injection achieved cures at a much higher rate than mice treated with EBRT or DC alone in a xenograft (nonprostate) flank-tumor model. Furthermore, these mice rejected repeat challenges of tumor re-implantations as opposed to

mice in the other treatment groups who readily accepted new tumors, suggesting that intratumoral DC injection in conjunction with irradiation also induced memory immunity [10]. Indeed, this study also demonstrated the induction of T cells specific for proteins that are prostate tumor antigens [10] with chemotherapy-induced apoptosis demonstrating similar synergy with DC administration [11].

Similarly, Candido and colleagues used a murine model to demonstrate DC injections into tumors with significant amounts of apoptosis (induced by injection of the protein TNF- α , not by irradiation) could result in a mononuclear cellular infiltrate. This induced a subsequent inhibition of tumor growth that directly correlated to CD8⁺ T-cell penetration [12]. Gulley and colleagues, working in a clinical trial, also described intraprostatic immunotherapy, with a viral vector, coordinated with curative-intent radiation therapy [13]. Techniques of clinical trials with intraprostatic injection of viral vaccine products - not autologous cellular products — have also been described previously [14–16].

We present here an initial human experience in patients with prostate cancer with autologous intratumoral DC injection coordinated with radiation therapy. The intent is to induce measurable specific anti-tumor immunity, while not interfering with definitive-intent local radiation therapy. Three broad concepts were the foundations for development of the approach:

- Provision of tumor-related antigens from the apoptotic bodies [8,10] of the hormone-deprived, irradiated, tumor-bearing prostate, which should be a pro-immunity environment [4];
- Androgen-suppression appears to be at least noninhibitory or potentially pro-inflammatory [17,18] and the noninvestigational part of the radiation approach is used without modification;
- Application in the clinical context in which if there is any distant tumor spread, it is microscopic in comparison to the bulk of the tumor that will be eliminated from the prostate and environs in the course of the primary tumor treatment.

Furthermore, this represents the first attempt to quantify timing of effector cell infiltrate prior to, during and after radiation therapy, at the times that would be coordinated with the novel method of intratumoral DC injection in man. To our knowledge, there are few data available to date *in vivo*, in human due to practical limitations on research biopsies obtained during treatment without direct clinical benefit.

Materials & methods

Patient selection & risk stratification

Adult male subjects participating in the trial were required to consent using the University of South Florida Institutional Review Board-approved informed consent document. Prostate cancer demonstrated by biopsy, absent evidence of metastatic disease by bone scan and CT scan of the pelvis (or other relevant radiological testing as clinically indicated), with disease features (clinical T stage, Gleason score and PSA) meeting the risk criteria for 2-year recurrence risk of 30% or more referring to the table as compiled by D'Amico *et al.* [19]

were required; one subject was later identified to have a slightly lower risk. Note that this study uses higher radiation doses than those used in the D'Amico cohort and that conventional follow-up for recurrence is for periods of time longer than 5 years. Eligibility was restricted to only HLA-A2⁺ subjects, assessed by low resolution antibody testing. Only a limited set of test peptides, specific for the HLA-A201 MHC I molecule were used, so this restriction accommodated a uniform testing approach. Any of the following were considered exclusionary: active urinary tract infection, metastatic disease, prosthetic joints, heart valves or vascular grafts, ongoing anticoagulation or bleeding disorder, prior pelvic radiation therapy, HIV or other primary immunodeficiency, or ongoing therapy with immunosuppressant drugs.

Dendritic cell preparation

A single pretreatment autologous leukapheresis product (~100 ml containing $\sim 1 \times 10^{10}$ mononuclear cells) was collected, aliquoted, frozen and stored in liquid nitrogen freezers at -150°C and -196°C . To prepare DCs, an aliquot was thawed, cultured in X-VIVO 15 medium (Cambrex, NJ, USA) on tissue culture flasks (Corning). Plastic adherent cells were cultured for 3 days in the presence of GM-CSF (Genzyme is the current manufacturer, MA, USA) and IL-4 (R&D Systems, MN, USA). Quality control testing included antimicrobial testing (Gram's stain, endotoxin testing, mycoplasma testing, bacterial and fungal culture) and flow cytometry testing (HLA DR⁺ and negative for lineage markers, referring to phycoerythrin-conjugated antibodies against CD3, CD14, CD19 and CD56 (BD Pharmingen, NJ, USA) [20]. Either 5×10^6 (subjects 1, 2 and 3) or 10×10^6 (subjects 4 and 5) cells resuspended in 1 ml of Plasmalyte-A solution (Baxter, IL, USA) and prepared for injection on radiation therapy days 5, 19 and 33, corresponding to alternate Fridays, after dose numbers 5, 15 and 25.

Conventional therapy for prostate cancer

Hormone therapy commenced with continuous GnRH agonist commencing between 44 and 30 days before start of radiation treatment and was planned to continue for 24 months of suppression [21], with concurrent bicalutamide 50 mg therapy taken orally once a day. The plan was to continue on GnRH agonist suppression through 24 months, although further treatment was at the discretion of the treating physician. Radiation treatment planning, including prostate volume assessment, was specified by the treating radiation oncologist, incorporating 4500 cGy into 25 fractions of 1.8 Gy, to a field for including the prostate and, at the discretion of the treating physician, pelvic lymph nodes, for 5 weeks of consecutive weekdays followed by an interval of 3–4 weeks. This was followed by the interstitial brachytherapy portion of the treatment. That was prescribed with I-125 seeds and 110 Gy D90 dose, or with Pd-103 seeds and a D90 dose of 90 Gy.

DC intraprostatic injection

Following the fractions 5, 15 and 25 of EBRT (always on Fridays) the autologous DC preparation was injected using 20 gauge spinal needle by the treating urologist (Seigne J and Pow-Sang J). The timing was based on the theory that DCs would take up apoptotic material in the irradiated prostate and migrate to regional lymph nodes (outside the radiation field) over the course of the 72 h in between treatments. Planned study-specific biopsies were

planned prior to the treatment and coinciding with the three injection dates and at 12, 24 and 36 months post-treatment. The coordinated conventional, injection and biopsy schedule is illustrated in Figure 1.

Clinical & immunological follow-up

Treatment-related toxicity was evaluated by postinjection follow-up phone call. The efficacy was evaluated with serial PSA tests, covering quarterly tests over 2 years. Follow-up for the patients is presented at 5 years.

Biopsy specimens from pretreatment, after fractions 5, 15 and 25 and at >3 months, biopsies were stained for hematoxylin and eosin stain, CD4⁺, CD8⁺ and apoptosis (scored as cleaved caspase 3 nuclear staining). Infiltrates were scored by a single pathologist (Kang L), blinded to temporal sequence as absent (0), sparse or focal (1+), moderate (2+), or dense (3+).

Blood was collected at the start of androgen blockade and on days 1, 15 and 29 from radiation start. Immune response was assessed by enzyme-linked immunosorbent spot (ELISPOT) for IFN- γ production by lymphocytes (200,000 cells/well, triplicate wells) with stimulation by synthetic peptides with sequences derived from proteins associated with prostate cancer for which there are HLA-A201 specific peptides: prostate-specific membrane antigen (PSMA, amino acids 441–450, LLHETDSAV and 711–719 and ALFDIESKV, used together); PSA (amino acids 166–175, FLTPKKLQCV), HER2/neu (amino acids 369–377, KIFGSLAFL); p53 (amino acids 264–272, LLGRNSFEV; the control peptide from influenza matrix M1, amino acids 58–66, GILGFVFTL, also HLA-A201 associated (custom synthesis; Synpep, Dublin, CA). Leukocytes from specimens from different time points were isolated, frozen and then thawed and tested on the same plate. Cell count was 200,000 lymphocytes per well, triplicate wells; 10 mg test-peptide per well (for each peptide when two were used) and other procedures as previously described [8,9].

Results & discussion

Patient characteristics, outcomes & feasibility

Between 2003 and 2005, 14 subjects consented to participate; of these, five were treated, five were ineligible because of absence of HLA-A2(+) and four were ineligible because of staging showing nonlocalized disease or for patient decision to not participate. Table 1 shows clinical features for the five treated subjects, including the clinical T stage, biopsy Gleason score and PSA and percentage for the 2-year probability of biochemical relapse predicted by as in Table 3B of D'Amico *et al.* [19]. All subjects completed 45 Gy EBRT treatment on schedule with no interruptions and no brachytherapy seed placement complications were identified.

PSA recurrence-free survival and follow-up are in the last column of Table 1. The size of the sample as well as the differences of the radiation technique preclude any direct comparative assessment of the recurrence frequency versus the tabulated experience of D'Amico *et al.* [19]. The clinical outcomes appear favorable, with zero of four assessable with recurrence at the 2-year time point. Given the small sample size, differences of radiation and hormone therapy and the nonrandomized design, further larger clinical testing is required before any

realistic assessment of generalizable clinical relevance can be made. Later outcomes are in Table 1.

Leukapheresis and DC preparation was completed as referenced in the ‘Materials & methods’ section above, with no instances of contaminated or unusable product. Cell counts for injection were as indicated above. Pain scores evaluated in the days following the injections were uniformly mild or none (data not shown). There were no dose-limiting toxicity events. Dose escalation was finished at the point of demonstration of immune response events as described below.

The intraprostatic DC injection was feasible and safe. This is consistent with the injection experience of other intraprostatic immune approaches [14–16]. Intratumoral DC injection with this technique of coordinated irradiation and introduction of autologous, unmanipulated DCs has been subject of further development in sarcoma therapy [22–25,102] and with KLH-pulsed DCs in pancreatic cancer therapy [103]. Other methods of intraprostatic DC injection coordinated with local therapy have been implemented a well, such as cryotherapy [104].

Histologic assessment: infiltrates & apoptosis

Prostate biopsies before treatment, after fractions 5, 15 and 25 (first, second and third injections) and 60 days after the third injection consistently showed low levels of infiltrates of either or both CD4⁺ and CD8⁺ cells. The later time points’ specimens were also tested for prostate cancer recurrences of which one biopsy in one patient showed <5% cancer present at 18 months; still consistent with a complete response.

There was not an obvious temporal pattern of induction of infiltrates by the radiation or the DC injection. Figures 2 & 3 shows representative infiltrates from five sequential time points for subject 1.

Theoretically, the optimal time to introduce the DCs would be when there is an apoptosis-rich environment. To address this timing issue, we assessed the percentage of tumor cells showing apoptosis at the time point of the injections. Among the 11 assessed cores, there were up to 19% of tumor cells showing apoptosis. The small percentage of apoptosis measured reflects the lack of ability to target tumor with the biopsies (the organ is targeted, not the tumor). Thus, we concluded that identification of a point, or points in time where radiation and testosterone induce a high level of apoptosis is not straightforward, but that there was ongoing apoptosis. Tables 2 & 3 show quantitation of the CD4 and CD8 infiltrates and observed apoptosis. The levels were uniformly low, but the pattern (Figure 3) shows the lymphocytes along the parenchyma, consistent with homing to cells that may bear relevant cancer antigen.

Quantitation of immune response

The ELISPOT testing demonstrated an apparently induced elevation of titers to at least one of the tested epitopes in two of the five subjects, for at least one time point after the first. Subject 1 had the biggest differences in PSA, PSMA and Her2/neu and a smaller increase in p53 peptide response. Subject 2 had generally higher initial titers, including some elevated at

baseline and almost all higher for the peptides than the control. Subject 3 appeared to have a nonspecific increase in IFN- γ -producing cells at the third time point, but without the specific increase as for the other subjects, including evaluation at the other time points. Subject 4 had no tested peptides that were clearly higher than the control, but the last two evaluations showed nonspecific increase versus the first three. Subject 5 had a baseline relative elevation of responses to PSA, PSMA and Her2-Neu, but did not have subsequent further elevation versus the control well. These data are illustrated in Figure 4.

Although there was at least one significant response to at least one specific antigen in four subjects, no consistent temporal pattern was evident. The relative importance of circulating versus infiltrative lymphocytes is not addressed by testing just the circulating leukocyte pool. However, it is reasonable to conclude that an abrupt or massive lymphocytic infiltrate during the treatment was not seen. Latent memory responses were not assessed. The potential therapeutic effect of more slowly developing immunity by impacting any cancer burden that was left after the initial definitive therapy on the tumor cells within the radiation volume is potentially of more clinical importance than any obvious immediate infiltration. This highlights a difference between the murine model system [6], in which a radiation dose that was suboptimal for cure of the local tumor volume and there were still viable tumors within the treated volume versus this clinical situation. In the clinical situation, the prostate irradiation is typically curative in the local sense, even for those subjects with later recurrence. The phenomenon of in-field recurrence of prostate cancer, in the absence of distant disease, is rare.

Some detectable lymphocytic infiltrates were observed in biopsy specimens. The temporal pattern of the induction did not suggest an obvious way that injection timing that could be modified to augment a response. The time until the onset of the effect (latency of either intraprostatic infiltrate or increased CTL titers) was variable between patients. In addition, the antigens for which there were observed increased CTL titers were variable between patients and between time points.

Conclusion

This pilot study could not address the question of whether radiation in combination with androgen ablation, without DC injection, could produce observed immune responses. However, Gulley *et al.* [13] observed in a randomized trial that in the absence of additional treatment besides hormone suppression and radiation, the acquisition of elevated ELISPOT reactivity to PSMA antigen did not occur in five of the six subjects receiving radiation therapy alone. More trials would be necessary to address the question of how each component of the therapy contributes to the immune events.

In recent years, the specter of late-recurrence after definitive-intent therapy of prostate cancer has been addressed with a variety of innovations focused on escalating local therapy, with higher radiation doses and surgical innovations. This window of opportunity for an immunological attack is not unique to prostate cancer. The approach here uses only autologous DCs, prepared in an in-house cell therapy facility and the microenvironment of the regression of the primary tumor is of distinct theoretical appeal. Although this pilot

study, emphasizing feasibility, safety and exploration of immune responses was limited to subjects with the *HLA-A0201* allele, in terms of efficacy testing, no HLA-restriction would be required. The present experience is quite limited in identifying a DC dose for further development; this may be a relevant factor.

Some key features that correspond to the model developed from the murine experiments were observed, notably induction of apoptosis during the course of the radiation, at time points corresponding to the DC injection and instances of increasing titers of CTL with specificity for cancer- or tissue-specific epitopes. Not surprisingly, no adverse events attributable to immune dysregulation were observed in this small series (data not shown) [26].

Human tumors bear multiple-potential rejection antigens. No proprietary or synthetic antigens are used for the therapeutic DC preparation. The panel of peptides used for CTL testing here was only five, representing four proteins; a bigger panel could have tested this issue with more generality. Establishing a connection between immunological correlates, including ELISPOT response and eventual impact on the eventual relapse-rate of the cancer remains unknown and this pilot trial does not and cannot address this. Ultimately, the gross tumor that is to receive curative intent radiation dose is not the key target — the therapeutic target is microscopic, metastatic disease, which is poorly assayed by any means except long-term follow-up.

The question of when to introduce DCs so as to provide the ideal stimulatory environment is complex. Both disease-specific and treatment-specific factors may vary widely across the potential treatment population. Serial biopsies within the parenchyma of the treatment volume showed an irregular pattern, despite a uniform treatment plan. Thus, there is not an obvious way to define an optimal time point for intratumoral DC injection. Brachytherapy, high-dose rate radiation therapy, high frequency ultrasound or cryotherapy could be other locally directed modalities that could be amplified by an immunotherapy approach. A different fraction size could yield a more predictable apoptosis pattern, which could affect when to introduce DC so as to get best uptake of antigen and a stimulatory microenvironment. This may be a key direction for development.

Future perspective

In summary, this initial translational experience with autologous DC injection, coordinated with EBRT of cancer showed safety and tolerability. Furthermore, we observed some increased titers of CTL with specificity for tumor antigen and it may induce lymphocytic infiltration into the tumor. The paradigm may be applicable in prostate cancer as well as in other clinical situations [22–28] where the entire grossly identifiable tumor volume is being treated primarily with radiotherapy and where that volume is accessible for intratumoral injections of autologous DCs. Newer radiation or intratumoral treatments may define situations suitable for clinical investigations of coordinated immunotherapy so as to get an optimization of when introduction of autologous DC when therapy-induced apoptosis is maximal.

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Executive Summary

Clinical needs & immunological opportunity

- Prostate cancer treatment with definitive intent radiation therapy may nonetheless relapse, more often for high-risk cases.
- This relapse may be an outcome of small volume metastatic disease present at the point of diagnosis.
- Placement of dendritic cells into apoptotic tumor can induce systemic immune anticancer responses, in murine models.

A novel schedule of radiation, hormone therapy & immune treatment

- Men with localized cancer with high-risk features, with HLA-A2 to accommodate testing with HLA-A2 associated peptides participated.
- The treatment schedule involved 28 months of androgen suppression, 45 Gy of external beam radiation, intraprostatic dendritic cell injection and brachytherapy seed placement.
- Interval biopsies and blood tests were used to assess immune reaction.

Feasibility & immunogenicity

- The coordinated treatment was well tolerated, as were the biopsies, in the five subjects.
- Biopsies showed some apoptosis and some limited lymphocytic infiltrates, including CD8⁺ cells.
- Tests on circulating lymphocytes showed some instances of increased titers to the test peptides.

Intratumoral dendritic cell injection is feasible for development

- Localized prostate cancer has ideal features for nontoxic, immunologically based complementary escalation, such as intratumoral dendritic cell injection.
- Careful development of coordinated apoptosis-inducing therapy with dendritic cell introduction merits further clinical study for prostate cancer or other oncologic application.

Figure 1. Treatment and testing schema

Brachy: Interstitial brachytherapy seeds; Bx: Biopsy; DC: Dendritic cell injection; E: Blood for enzyme-linked immunosorbent spot testing; EBRT: External beam radiation therapy.

Figure 2. Immunohistochemistry for CD4, CD8 and cleaved caspase (apoptosis) at five time points, illustrated for one subject

Figure 3. Parenchymal distribution of infiltrating CD8⁺ lymphocytes
Apoptotic tumor cells can also be seen.

Figure 4. Enzyme-linked immunosorbent spot results

IFN- γ -producing spots per 200,000 cells/well after stimulation with 10 μ g peptide. No p-values are computed. The error bars are fixed at 20%, estimated from the overall enzyme-linked immunosorbent spot test experience. Light bars represent control peptide and (A-E) represent subjects 1–5, respectively. +n d: Time point after end of external beam radiation (day); +n mo: Time point after end of external beam radiation (month); fx 5/10/15: Time point after the fractions 5, 15 or 25 of external beam radiation; Pre: pretreatment; PSA:

Prostate-specific antigen; PSMA: Prostate-specific membrane antigen; x: No data for a time point.

Table 1

Clinical features, risk estimates and follow-up.

Subject	Stage (cN0cM0)	Gleason score	PSA	2-year recurrence (%) [†]	Follow-up: time to detectable PSA
1	cT1c	9	6.96	33 (20–50)	PSA recurrence at 49 months
2	cT2c	7	151	51 (38–66) [‡]	PSA recurrence at 57 months
3	cT2b	7	9.6	26(17–37)	PSA(–)at 15 months (no further data)
4	cT2a	9	8.34	40 (24–59)	PSA (–) at 25 months (no further data)
5	cT1c	9	10.0	33 (20–50)	PSA (–) ongoing response at 62 months

[†] By Table 3B of [18].
[‡] Computed using PSA: 50 Instead of 151.
PSA: Prostate-specific antigen.

Table 2

Quantitation of therapy-related changes and apoptosis among visible tumor cells within the biopsy and CD4⁺ or CD8⁺ infiltrates among 22 evaluable specimens.

Time point (apoptotic tumor cells %)	Subject								
	1	2	3	4	5				
Baseline biopsy	+	9%	-	3%	2%	m	19%	m	NTS
At fraction 5	+	9%	+	NTS	6%	m	9%	+	5
At fraction 15	+	16%	+	NTS	+	NTS	ND	+	5
At fraction 25	+	2%	+	NTS	+	NTS	ND	-	NTS
>3 months later	+	NTS	+	NTS	+	NTS	ND	ND	ND

-: Absent; +: Therapy-related changes; m: Minimal; ND: No data; NTS: No tumor cells seen.

Table 3

Quantitation of therapy-related changes and apoptosis among visible tumor cells within the biopsy and CD4⁺ or CD8⁺ infiltrates among 22 evaluable specimens.

Time point	Subject 1		Subject 2		Subject 3		Subject 4		Subject 5	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Baseline biopsy	1+	1+	1+	1+	1+	1+	1+	1+	0	1+
At fraction 5	1+	1+	1+	1+	1+	1+	0	1+	0	1+
At fraction 15	1+	1+	1+	1+	0	0	ND	ND	0	1+
At fraction 25	1+	1+	0	1+	1+	1+	ND	ND	1+	1+
>3 months later	1+	1+	0	0	1+	1+	ND	ND	ND	ND

CD4, CD8 infiltrates: 0/1+/2+/3+.
ND: No data.