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1 Introduction

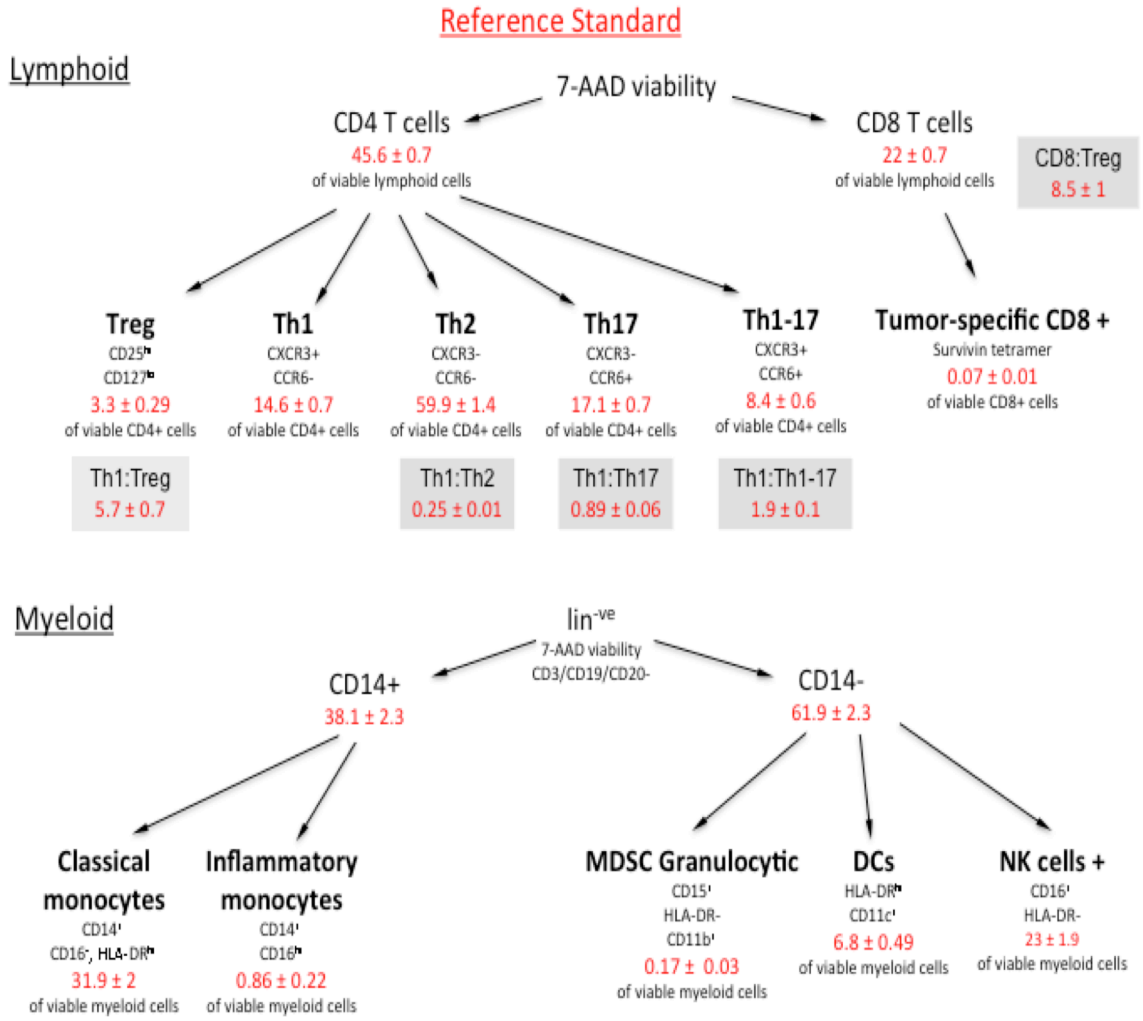
This study combines the TGFbeta neutralizing antibody, Fresolimumab, with Radiation Therapy (RT) to treat advanced metastatic breast cancer. Fresolimumab was given 5 times as either 1 mg/kg or 10 mg/kg 3-weekly schedules. RT is limited to IGRT (3 x 7.5 Gy) to 1-2 lesions with other lesions being designated as sentinels to determine abscopal responses that are hypothesized to be due to RT-induced vaccination. RT was started 10 days after the first and 3rd dose of Fresolimumab

The primary objectives are to assess safety, feasibility, and abscopal tumor regression and to monitor immune responses in these patients. The abscopal responses are assessed by imaging. The UCLA component is 3 fold: 1) to enroll 15 patients into the clinical trial, 2) to assess immune responses using blood samples before, during and after treatment by multi-channel flow cytometry for immune monitoring, 3) to examine the effects of targeting TGF-beta on the activities and numbers of breast cancer stem cells with and without irradiation.

2 Body

2.1 Immune-monitoring

Figure 1: Immune monitoring of myeloid and lymphoid cells with control values shown in red.



At UCLA, 8 patients have been enrolled and we have embarked on a major effort to enroll the final 7 patients as soon as is feasible.

With help from the DOD, we have replaced our flow cytometer that had laser problems with a more powerful 18 color, 4 laser BD Fortessa. New reagents have been optimized to obtain correct discrimination. Quality control parameters have been established for this machine and its ability to match values to values obtained previously has been confirmed. We are very pleased by its performance. The multicolor flow cytometry panel that has been developed (Fig. 1) for lymphoid and myeloid cells has been "tweaked" to exploit the greater power that the Fortessa affords.

We have monitored samples from 16 patients from UCLA and NYU cohorts, although not all had finished the full 15 weeks of treatment. Nine of these were HLA-A2.1 positive and were assessed for responses to the tumor-specific antigen survivin

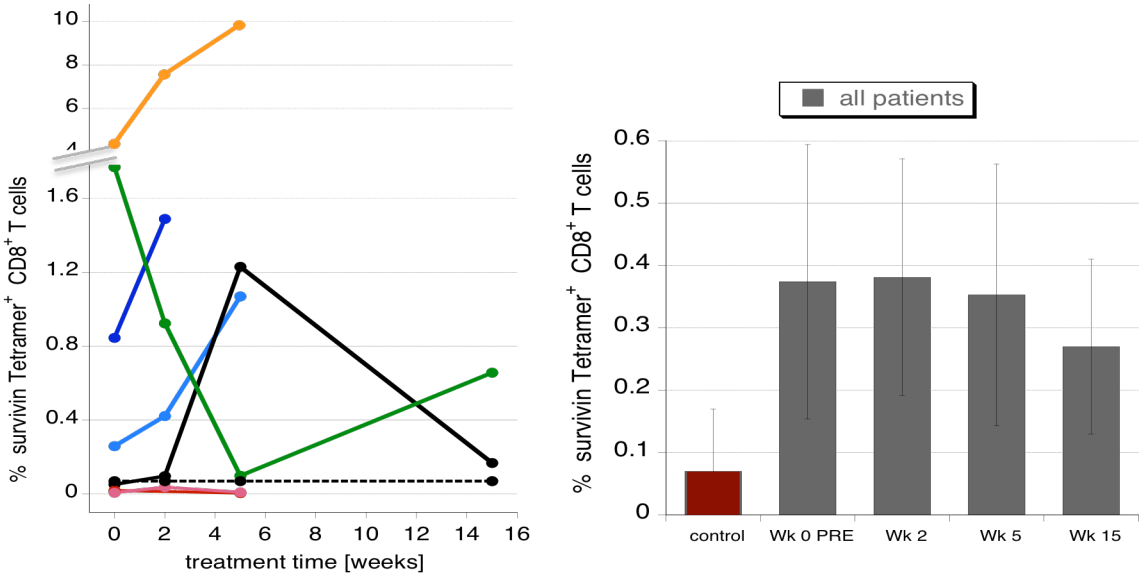
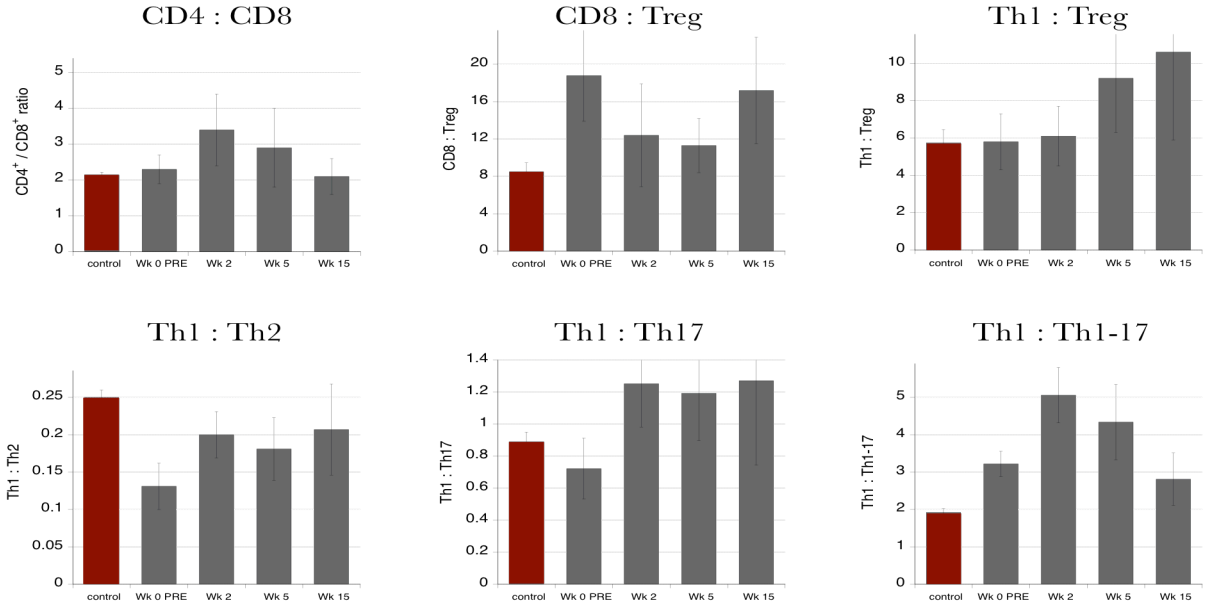


Figure 2: CD8+ survivin-specific T cells levels with time after initiation of treatment. Individual NYU patients (left) and average values for all patients +/- 1 SEM (right).

Figure 3: Alterations in T cell ratios with time after Fresolumimab and RT



before and after Fresolumimab and RT (Fig 2). Four had prexisting CD8+ T cells that recognized survivin. Five responded to treatment with an increased level of anti-tumor activity, 2 of whom were previously non-responders. This is highly encouraging. In one patient, a strong pre-treatment level decreased with time. Three did not respond.

Changes in the ratios of T cell subsets after Fresolumimab and RT are shown in Fig 3. There are clear indications of major changes after Fresolumimab alone (week 2). Overall, the CD4:CD8 ratios were higher 2 weeks after the start of treatment than pre-treatment before becoming "normal". This was due to dramatic increases in 3 out of 14 patients, and primarily to an increase in the Th1 subset (not shown). Th2 cells tended to be high in patients. Resulting in a low Th1:Th2 ratio that was also altered by Fresolumimab alone. Th17 cells generally decreased after Fresolumimab with a resultant large change in Th1:Th17 ratios (Fig 3).

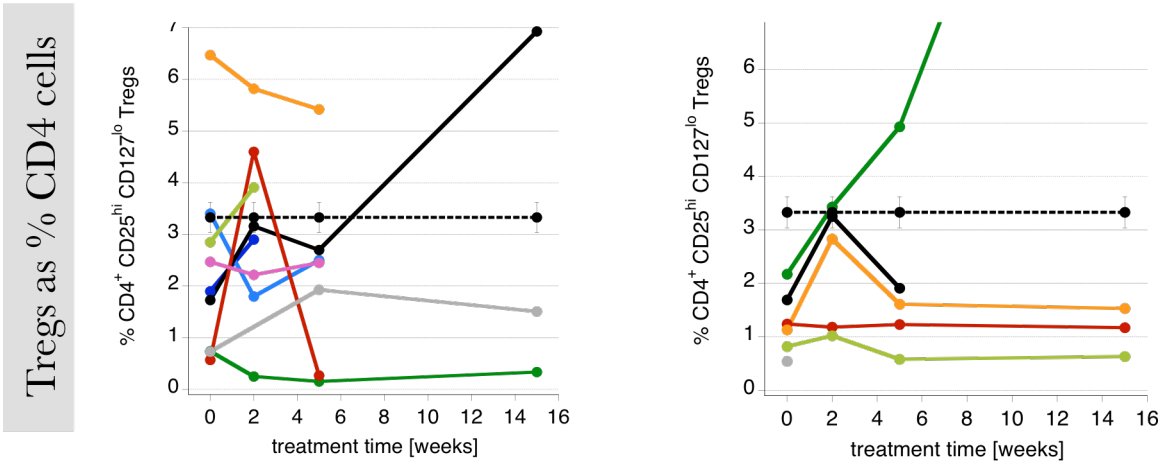


Fig 4: Treg cells as a % of CD4+ cells. Similar data were obtained for % of all lymphocytes.

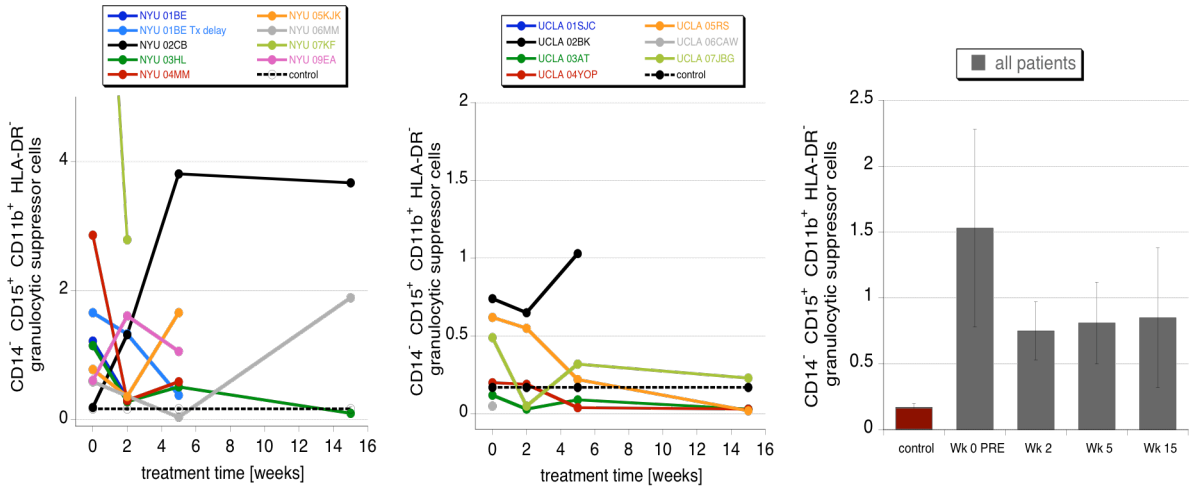


Figure 5: Cells of the myeloid suppressor cell lineage before and after treatment.

Levels of Treg cells were almost universally, unexpectedly low, with only one exception. After one dose of Fresulimumab, Treg levels increased in about 75% of

cases before falling again after the start of RT. Two patients out of 7, for whom samples were available for the whole treatment course, showed dramatic late increases in Tregs (Figure 4).

Myeloid cell subsets were evaluated in depth. The most interesting finding was that cells of the myeloid-derived lineage that are generally accepted to be immunosuppressive, were high in about 70% of patients and almost universally fell after Fresolumimab treatment, suggesting that TGF-beta may be responsible foir maintaining this subset (Fig 5). One patient, however gave an opposite result with dramatic increases with time, starting from a low "normal" level.

2.2 Effects of TGF-beta on breast cancer stem-cells

Personnel change in this part of the project, as Chann Lagadec returned to his native France and his place has been taken by Erina Vlashi.

Recent preclinical and clinical data support that solid cancers including breast cancers are organized hierarchically with a small population of cancer stem cells (CSCs), capable of re-growing the entire tumor while their progeny lack this ability. We and others reported that breast CSCs (BCSCs) are relatively resistant to ionizing radiation and after irradiation, the surviving BCSCs are recruited from a quiescent state (G0) into an active cell cycle, allowing repopulation of the tumor. Furthermore, we have shown that RT can induce reprogramming of non-tumorigenic cancer cells to generate new cancer stem cells (induced cancer stem cells, iCSCs). The mechanisms involved require the re-expression of the stem cell transcription factors Oct-4, Sox2 and Nanog. Interestingly, this re-expression was higher in polyploid cells.

TGFβ activation is a regulator of BCSCS expansion and the project aims at investigating its effect on reprogramming of non-BCSCS into BCSCs. Interestingly, we have observed varying effects for BCSCs when breast cancer cells were treated with an inhibitor of the TGFβ receptor. Several schedules have been investigated using 3 different cell lines. TGF-β seems to inhibit reprogramming of SUM159PT, a claudin-low cell line, while it enhances reprogramming in MCF7, a luminal cell line. MDA-MB-231, a basal cell line, was very sensitive to TGF-βR inhibition resulting in significant toxicity. These divergent data that may depend upon the origin of the breast cancer are important if we are to further understand the effects of Fresolimumab and RT on BCSCs and this work is ongoing.

This aspect of the project has resulted in numerous publications over the last 2 years.

1. Lagadec C., Vlashi E., Della Donna L., Dekmezian C. and Pajonk F., *Ionizing Radiation Reprograms Non-tumorigenic Cancer Cells into Cancer Stem Cells*. **Stem Cells**, 2012, May;30(5):833-44

2. Lagadec C, Dekmezian D, Bauché L, Pajonk F, *Oxygen levels do not determine radiation survival of breast cancer stem cells.* **PLOSone**, 2012; 7(3): e34545

3. Steinberg ML, McBride WH, Vlashi E, and Pajonk F, *NIH funding in Radiation Oncology – A snapshot.* Int J Radiat Oncol Biol Phys. 2013 Jun 1; 86(2):234-40

4. Pajonk F. and Vlashi E., *Characterization of The Stem Cell Niche and Importance in Radiobiologic Response.* Seminars in Radiation Oncology, 23(4), 2013, p237-41

5. Lagadec C, Vlashi E, Phillips TM, Chann M, Frohnen P, and Pajonk F, M.D./Ph. *Radiation-Induced Notch Signaling in Breast Cancer Stem Cells.* IJROBP, 2013, epub ahead of print

6. Lagadec C, Vlashi E, Frohnen P, Alhiyari Y, Chan M, and Pajonk F, *The RNA-binding protein Musashi-1 regulates proteasome subunit expression in breast cancer and glioma CSCs/tumor-initiating cells,* Stem Cells. 2013, in press

7. Vlashi E, Lagadec C, Chan M, Frohnen P, McDonald AJ, and Pajonk F, *Targeted Elimination of Breast Cancer Cells with low Proteasome Activity is Sufficient for Tumor Regressio.,* Breast Cancer Research and Treatment, 2013, epub ahead of print.

3. Problems Encountered

There have been no problems with subject enrollment or retention. We have received all subjects from the UCLA Department of Hematology-Oncology as referrals. There are no problems to report regarding the conduct of study procedures, consenting, confidentiality or anything else that would be considered reportable. All SAEs and AEs have been reported to NYU and the UCLA IRB as per protocol.

4. Future directions

We have started to evaluate several future directions in collaboration with our colleagues at NYU. In addition to accumulating and testing more patient samples, we have the opportunity to build on the patient data that we have by performing another round of assays. To this end, we are evaluating if biopsy samples can be used to stain for alternative tumor-associated antigens. After we have examined the biopsy situation, we will determine the final course of action for the remaining samples. The options we are looking at are not mutually exclusive but will be designed to give the most relevant information for the study. They are to:

1. Extend the survivin epitopes to other HLA types.
2. Extend the range of tumor associated antigens to examine if there is a broad increase in other responses, or epitope “spreading.”
3. Perform ELISpot assays to determine specific anti-tumor activity in all patients.
4. Perform intracellular cytokine flow cytometry to evaluate functional aspects of the T cell response to tumor-associated antigens and non-specific stimuli. This would be of particular interest with respect to interferon-gamma for Th1 subsets and Th17 for the Th17 subset. We have shown that the ratio changes with time for these subsets following Fresolimumab treatment (see fig).
5. Extend the analyses to better evaluate NK cell subsets, which recent data suggest may be influenced by TGF-beta.

6. Examine the functionality of myeloid suppressor cells. A major finding was that this subset decreased by week 2 and remained low, consistent with the subset being driven by TGF-b. There was one marked exception. This patient converted from being negative for CD8+ anti-tumor activity to being very positive at week 5 before falling slightly by week 15. This increase in CD8+ tumor-specific reactivity was associated with a marked abscopal effect. The fall in CD8+ tumor-specific reactivity was associated with a major increase in Treg cells on week 15, with Th1 cells plummeting on week 5 and 15 to very low levels, and an increase in myeloid suppressor cells to very high levels at week 5 and 15. This patient developed marker-confirmed myelodysplasia, from which she died.

Currently, we are developing assays for intracellular cytokine staining and evaluation of functional suppressor cell activity. We are also examining NK cell subsets in normal control and cancer patient samples that are not part of the precious resources we have in this trial.

We are currently involved in obtaining Fresolumimab or in vitro stem cell work, having evaluated responses up until this stage with specific inhibitors of TGF-beta pathways. The comparison will be of considerable interest.

5. Key Research Accomplishments

- Trial recruitment is proceeding with 7 patients still to go.
- Immune monitoring is yielding interesting data, particularly in response to Fresolumimab alone.
- TGF-beta affects non-stem cell reprogramming by radiation exposure in a manner that depends on the origin of the brest cancer cell line.
- Numerous publications (see section 2.2).