CD4 T Cell-Driven Response to Immunotherapy Against Mouse B78 Melanoma Tumors

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BACKGROUND

- Using an in situ vaccine (ISV) regimen that includes a combination therapy of radiation (RT) together with immunocytokine (IC, a tumor-targeting mAb linked to IL2), we can cure mice of B78 melanoma tumors.
- During the antitumor response to ISV, T cells are involved in the antitumor response.

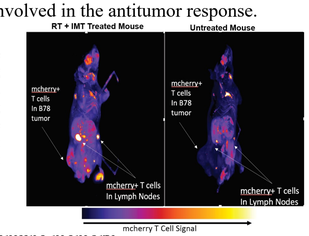


Figure 1. T cell presence in the tumor and expansion in the tumor-draining lymph node (TD-LNs) is observed following ISV. Using mice that have mcherry expression on their T cells, via whole mouse Cryo-Fluorescence Tomography imaging (Emit Imaging) we observe an influx of T cells in both the tumor and in the TD-LNs on Day 13 (D13) following treatment with ISV as compared to untreated mice, as we observed bright fluorescent signal in the inguinal LNs (both the TD-LN and distal inguinal LN) as well as within the tumor itself.

- B78-bearing mice cured via ISV have long-term immune memory.
  - Traditionally, immune memory is thought to be mediated via CD8 T cells, which require antigen presentation via MHC Class I (MHCI).
  - B78s expresses little to no MHCI but do express MHCII when stimulated with IFNγ.

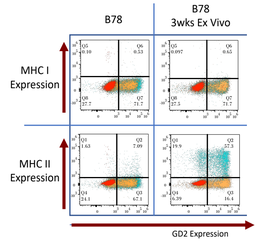
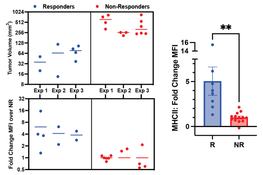


Figure 2. MHC expression on B78 melanoma. **A)** B78 cells plated with or without IFNg (200U/ml) for 48 hrs and assessed for GD2 expression and MHC-I or MHC-II expression via flow cytometry. B78s (left panel), which express GD2, showed no MHCI expression and some expression of MHCII when stimulated with IFNγ. Following in vivo passage, MHCII expression is increased following in vivo growth: A B78 tumor implanted in a mouse for ~4 weeks was excised, dissociated and then grown in vitro for ~3 weeks ("B78 3wks Ex Vivo", right panel). The B78 3wks Ex Vivo cell line still showed no expression of MHCI, but had enhanced expression of MHCII upon stimulation with IFNγ.

- Not commonly expressed on solid tumors, MHCII is expressed on 50-60% of melanomas in humans. MHCII expression on human melanoma may be associated with improved response to immunotherapy.

Figure 3. MHCII expression may influence response to ISV for B78 melanoma. B78 tumors extracted from mice following ISV regimen showed increased MHCII expression on mice that were responding to treatment (based on tumor shrinkage) as compared to tumors from mice that were not responding, suggesting that MHCII may be beneficial for the antitumor response to ISV in this model.



Here we explored the implications of MHCII expression on response, and how CD4 and CD8 T cells responses are driven in our translationally-relevant murine melanoma model.

METHODS

- **In Vivo Studies:**
- **Depletion during response to treatment:** Mice bearing B78 tumors (~100m³) were treated with our ISV regimen of RT (12 Gy, D0) and intratumoral immunocytokine ("IT-IC"; 50ug, D5-D9), and randomized into treatment groups. In combination with ISV, mice were not depleted and treated with control rat IgG, or treated with immune depletion of CD4 T cells, CD8 T cells or NK cells throughout the course of treatment (D-2 to D18). Tumor volumes were measured twice weekly.
- **Depletion during memory rechallenge:** Mice cured via the ISV regimen were rechallenged with B78 tumor cells ~30 days after developing a complete response. Mice were not depleted and treated with control rat IgG or treated with immune depletion of CD4 T cells, CD8 T cells or NK cells throughout the course of rechallenge (D-2 to D18). Tumor volumes were measured twice weekly.
- **Phenotypic Analyses:**
  - **Isoplexis:** IsoCode Single-Cell Adaptive Immune chips were used to assess cytokine release from CD4 or CD8 T cells within the TD-LNs of B78-bearing mice treated with ISV or NT (3 mice/group) or a naive mouse. TD-LNs were excised from mice on D8 following treatment, stimulated with CD3/CD28 for 30 hrs, and the Mouse T-cells Protocol was followed. chips were run on IsoSpark Duos.
  - **Flow Cytometry:** On D8 during the course of treatment, or on D7 following tumor rechallenge injection, tumors (for treatment flow) or lymph nodes (tumor rechallenge flow) were harvested and stained with antibodies. 4-5 mice/group.
- Other methodology described within figure legends.

RESULTS

Figure 4. CD4 and CD8 T cells are activated via ISV.

A) Isoplexis analyses of TD-LNs show that CD4 and CD8 T cells have similar phenotypes based on treatment.

B) Even though B78s do not express MHCI, CD8 T cells display a large increase in polyfunctionality with ISV treatment. CD4 T cells also increase their polyfunctionality with ISV compared to untreated mice.

C,D) The types of cytokines released from CD8 and CD4 T cells are largely associated with an effector function.

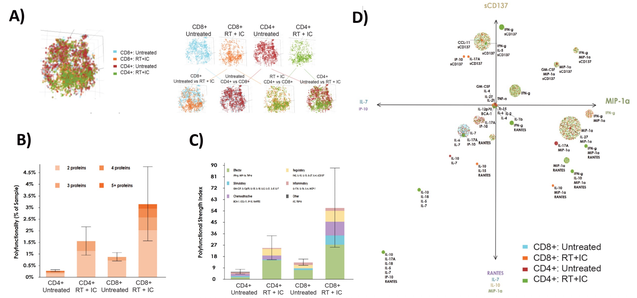
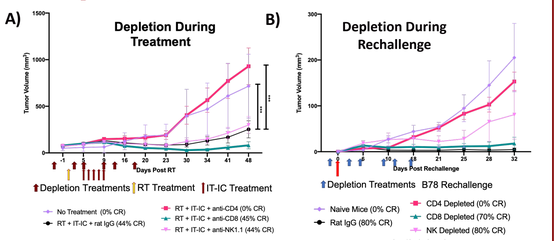


Figure 5. CD4 T cells are required for the antitumor response, CD8 and NK cells are not.

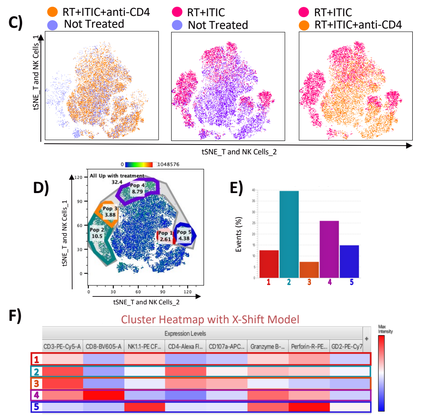
Depletion of immune cell subsets during the course of ISV **(A)** or during the memory rechallenge **(B)** reveals:

1) CD4 T cells are required for response.
2) Neither CD8 T cells (teal line) nor NK cells (light pink) were required, as mice depleted of CD8 T cells or NK cells respond similarly to mice not depleted of any immune cells (black).



C) Flow cytometry analysis of B78 tumors excised on D8 following treatment with ISV, no treatment, or ISV with CD4 depletion reveals:

1) ISV+CD4 depletion (orange) have a similar phenotype to tumors from untreated mice (purple) (left panel).
2) Compared to untreated mice (middle panel) or ISV+CD4 depletion (right panel), distinct, but similar, subsets of cells are upregulated with ISV (pink).

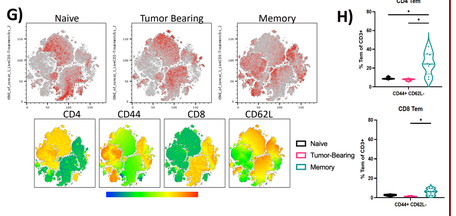


D-F) Unsupervised cluster analyses of most increased subsets following ISV (selected in gray in part **D**) reveals 5 separate cell populations (**E, F**). The subsets reveal:

1) CD4 T cells are included in Pops 2 and 3, and are the only subset that express CD107a.
   a) Pop 2, the most increased, has both CD107a expression with Granzyme B and Perforin.
   b) Pop 3 has the highest amount of CD107a.
2) Pop 1 (NKT cells), Pop 4 (CD8 T cells) and Pop 5 (NK cells) express Granzyme B and Perforin, but express minimal CD107a.
3) Pops 2 and 5 appear to have some GD2 expression.

G,H) Flow cytometry analysis of TD-LNs harvested on D7 (post-rechallenge) from mice that were naive (never injected with B78s), tumor bearing (injected with B78s but not treated), or memory (B78s cured by ISV and rechallenged) reveals:

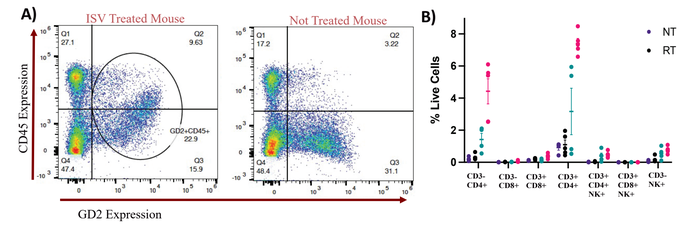
1) tSNE analyses show shifts in T cell subsets based on memory phenotype.
2) CD4 T effector memory cells (T_{em}) were significantly increased in both CD4 and CD8 T cells, with a larger subset of T_{ems} in the CD4 T cell subsets.



RESULTS

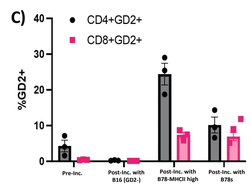
Figure 6. CD4 T cells trogocytose B78 tumor cells during the antitumor response.

Flow cytometry showed that following ISV, some immune cells become GD2+ **(A)**. Further analysis showed that CD4 T cells (i.e. CD3+CD4+NK-) have the highest population of GD2 expression **(B)**.



C) TD-LNs removed from mice bearing B78 tumors were incubated with B16s (which do not express GD2), B78s (which express GD2), or B78s that express high MHCII levels.

1) Prior to incubation, CD4 and CD8 T cells expressed minimal GD2
2) Post incubation with GD2- cells (B16s), LNs do not express GD2
3) Post incubation with GD2+ cells, GD2 expression was increased, with the most expression on MHCII+ B78s.

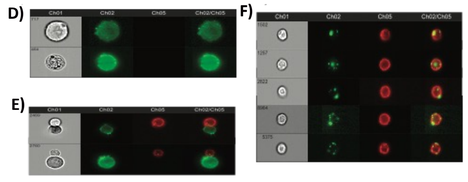


D-F) TD-LNs removed from B78-bearing mice on D8 following ISV. Tumors were dissociated, and live cells were stained with CD4 (red) or GD2 (green) analyzed on an ImageStream MarkII.

D) GD2+ tumor cells are large and have homogenous GD2 expression.

E) Few doublet cells were observed, with one cell having homogenous GD2 and one with homogenous CD4.

F) CD4 T cells had bright red signal, with punctate GD2 positivity on the surface, suggesting trogocytosis of GD2 tumor cells.



CONCLUSIONS AND FUTURE DIRECTIONS

Understanding the details of the cellular and molecular mechanisms involved in the B78 tumor-immune system interaction will guide future improvements of this clinically-relevant immunotherapy regimen.

- CD4 T cells play an important role in the antitumor response against this MHCII-expressing tumor when when tumors are ~100mm³.
- The expression of MHCII on B78s may influence the role for CD4s in this melanoma model, but more work needs to be done to address this.
- We are continuing to address the role of the immune cells and gene expression of the tumor cells that might influence the antitumor and memory responses in this B78 model (e.g., scRNAseq and sorted RNAseq analyses).

ACKNOWLEDGEMENTS

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