Tumor Microenvironment Characterization in a Preclinical Model of Head and Neck Squamous Cell Carcinoma (HNSCC) to Evaluate Antibody Therapies

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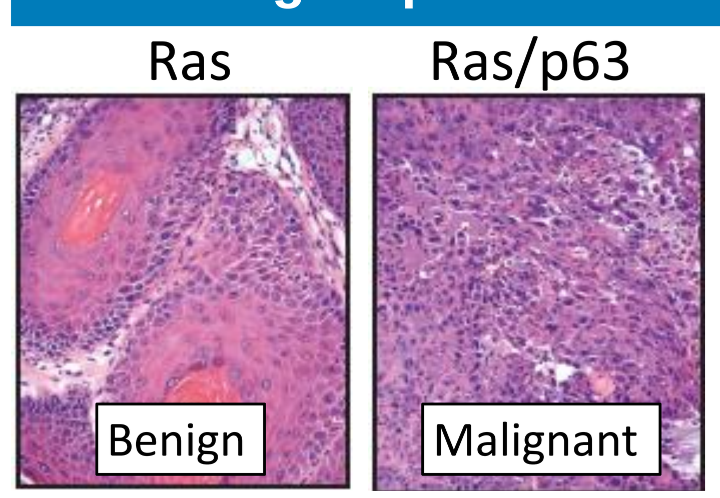
Abstract

Chromosome 3q is frequently amplified in squamous cell carcinoma (SCCs) of multiple tissue origins. The 3q arm encodes multiple proto-oncogenes including TP63, where the amplification results in overexpression of ΔNp63α, a predominantly expressed isoform of p63. Squamous cell carcinomas of the head and neck (HNSCC), frequently associated with the ΔNp63α overexpression, are also typically marked by heavy immune cell infiltrates. Both development and response to the therapies of these tumors reflect a balance between anti-tumor immune responses and mechanisms of immune evasion. Many of the oncological monoclonal antibody (mAb) products regulated by our office (Office of Biotechnology Products, OBP) rely on Fc domain effector functions to modulate the activity of immune cells in the tumor microenvironment (TME) to achieve clinical efficacy. Using HNSCC-derived cell lines, as well as an experimental model of HNSCC that employs primary murine keratinocytes and lentiviral vectors to model molecular alterations reported in HNSCC (e.g. p63 amplification and activated Ras), we previously established that overexpression of ΔNp63α protein enhances nuclear localization and activation of NF-κB/c-Rel, a known mediator of inflammatory responses. Furthermore, ΔNp63α cooperates with oncogenic H-Ras to drive malignant conversion of H-Ras-initiated papillomas in nude mice. Here, we established a grafting model in immune competent syngeneic mice to profile changes in the TME during malignant conversion. Resulting papillomas (H-Ras+) and carcinomas (H-Ras+/ΔNp63α+) were harvested 2-4 weeks post-grafting and analyzed. While no significant differences were observed in CD4+ T cells, CD8+ T cells, and regulatory T cells (Tregs) between tumor types at two weeks, we observed a significant increase in polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) in carcinomas compared to papillomas. These carcinomas express elevated levels of chemokines and their receptors associated with an immunosuppressive TME. Our data suggest that altered immune infiltrate by ΔNp63α/H-Ras-expressing carcinomas establishes an immunosuppressive TME to allow development of a malignant phenotype. The impact of TME components on mAb efficacy is currently under study.

Multi-stage squamous cell carcinoma model



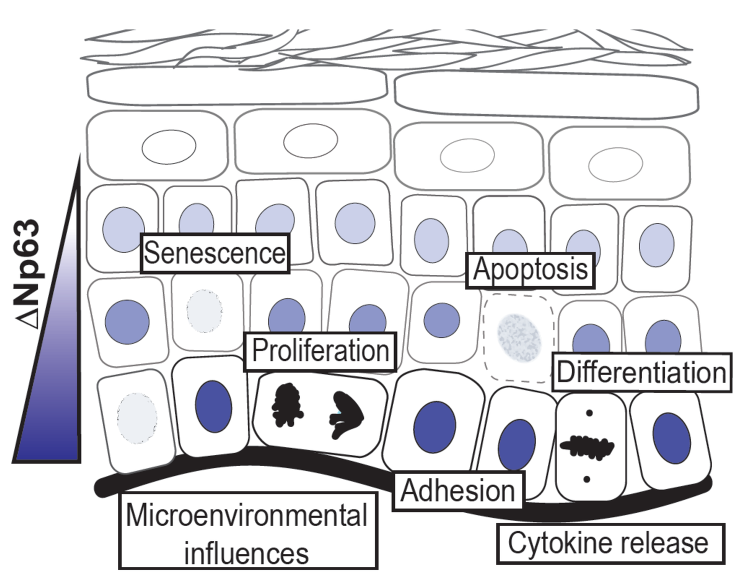
Ha et al., *PLoS ONE* 6(7): e21877, 2011.

Graft Model

- Grafting of Ras expressing keratinocytes induces papillomas
- Grafting of Ras/ΔNp63α (p63) expressing keratinocytes induces carcinomas

Role of ΔNp63α

- Known as a "Master regulator of epithelial cells"
- Dominant isoform expressed in basal cells of epithelia
- Regulates proliferation, differentiation, senescence, adhesion
- Often overexpressed in HNSCC and lung SCC
  - 3q amplification
- Immune modulator
  - Activates c-REL (NF-kB)



FDA mission relevance

Therapeutic antibodies constitute a large portion of the OBP portfolio, including oncology indications. Patient responses are dependent on the tumor microenvironment (TME) and modulated by antibody glycovariants. We defined immune infiltrates in a pre-clinical model of HNSCC to assess the impact of specific immune cells on clinical efficacy of mAb therapies.

Objective

To establish a pre-clinical model of multi-stage squamous cell carcinoma to define the immune microenvironment for optimizing quality attributes of mAb therapies.

Goals

1. Establish a syngeneic (immune-competent) mouse model (not shown here)
2. Identify immune cell infiltrates in tumor microenvironment (TME)
3. Analyze chemokines and cytokines in TME

This model will be used to evaluate mAb quality attributes in distinct tumor context

Materials and Methods

Grafting experiment:
The primary keratinocytes were transduced with viral vectors encoding oncogenic H-Ras and ΔNp63α and empty vector control (stuffer). After 9 days in culture, the keratinocytes and fibroblasts were grafted as described in (PMID: 21789189). The tumors and grafted sites were collected at 2 weeks post grafting.

Flow Cytometry:
Cells purified from tumor or spleen were incubated with CD16/32 (FcR block) antibodies for 10 minutes. Cells were stained with primary antibodies for 30 minutes. Anti-mouse CD45.2 (clone 104), CD11b (M1/70), Ly-6C (HK1.4), Ly-6G (1A8), CD3 (145-2C11), CD8 (53-6-7), CD4 (GK1.5), CD25 (PC61.5.3), FoxP3 (FJK-16s), NK1.1 (PK136) antibodies were purchased from Biolegend or eBioscience. 7AAD was used to determine viability and a "fluorescence minus one" method was used to determine antibody specificity. For intranuclear staining cells were fixed and permeabilized using a FoxP3 staining kit (eBioscience) per manufactures' protocol. All samples were analyzed on a BD FACSCanto analyzer using FACSDiva software. Post-acquisition analysis was performed using FlowJo vX10.0.7r2.

T Cell Proliferation Assay:
T lymphocyte proliferation assay was performed as previously described (PMID: 28364000). CD4 and CD8 T cells isolated from naïve B6 spleens were labeled with CFSE (Sigma) and stimulated with plate-bound anti-CD3 (clone 145-2C11, eBioscience) and CD28 (Clone 37.51, eBioscience) antibodies. T cells were cocultured with MDSCs isolated from spleens, or benign (H-Ras+) and malignant (H-Ras+/ ΔNp63 +) tumors. Flow cytometry was used to quantify 72-hour CFSE dilution. Proliferation was quantified as the average number of divisions of all cells in the culture (division index) using FlowJo software (PMID: 21265003).

Results and Discussion

Figure 1.

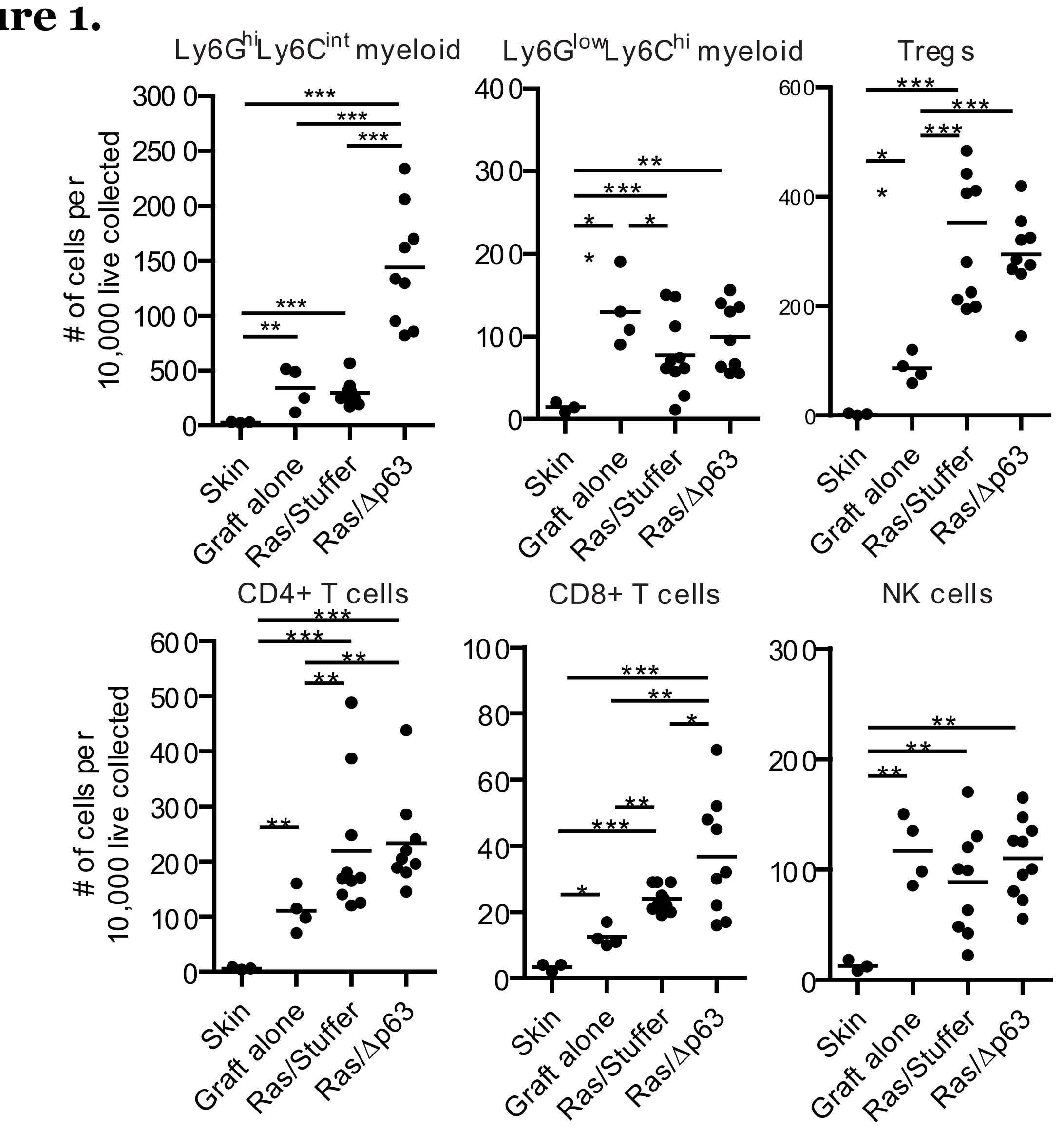


Figure 1. Significantly increased numbers of Ly6G[hi]Ly6C[int] myeloid derived cells are recruited in Ras/ΔNp63 carcinoma. Tumors were dissected and cells were identified using Flow Cytometry as described in the method section. Ras/ΔNp63-induced carcinomas recruited significantly increased numbers of Ly6G[hi] cells in the tumors compared to Ras-induced papilloma or graft or skin. Both benign and malignant tumors recruit elevated levels of Tregs and CD4+ T cells compared to skin or graft alone suggesting that oncogenic-Ras. Recruited CD8+ T cell numbers increase with wounding, and tumor phenotypic progression. Ly6G[low] myeloid cells and NK cells were recruited to both tumor phenotypes as well as to control grafts, indicating that the recruitment is independent of oncogene expression.

Figure 2.

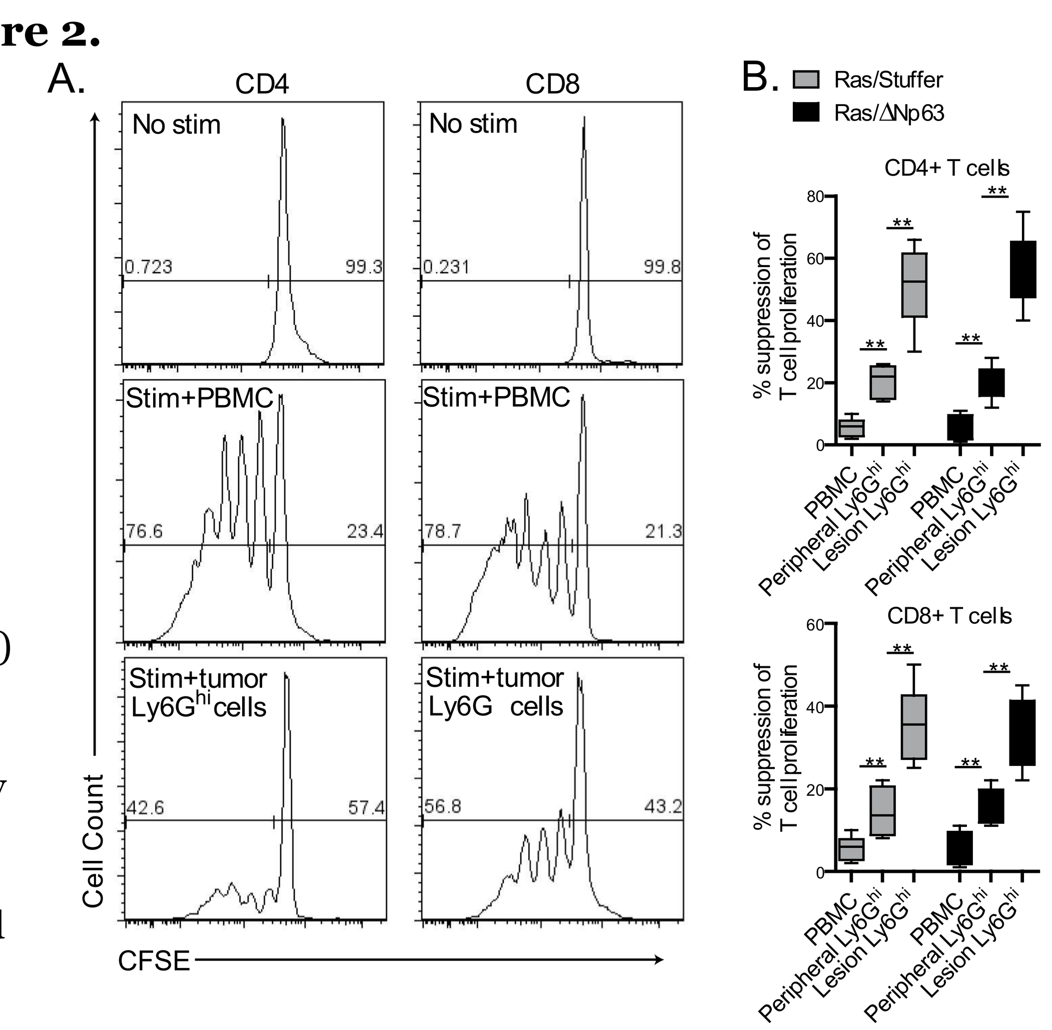


Figure 2. Ly6G[hi] cells isolated from tumors are immunosuppressive
To distinguish Ly6G[hi] polymorphonuclear-myeloid derived suppressor cells (PMN-MDSCs) from Ly6G[hi] neutrophils, Ly6G[hi] cells from tumors were isolated and assessed for their ability to suppress the proliferation of CD3/28 stimulated wild-type CFSE-labelled CD4+ and CD8+ T cells in comparison to total splenocytes (PBMC). Proliferation was assessed by flow cytometric analysis. A, representative CFSE histograms of unstimulated T cells (top panels) or T cells co-cultured with splenocytes (middle panels) or Ly6G[hi] cell isolated from a Ras/ ΔNp63 lesion. B, quantification of % suppression of T cell proliferation. Data pooled from three experimental replicates. **, $p < 0.01$.

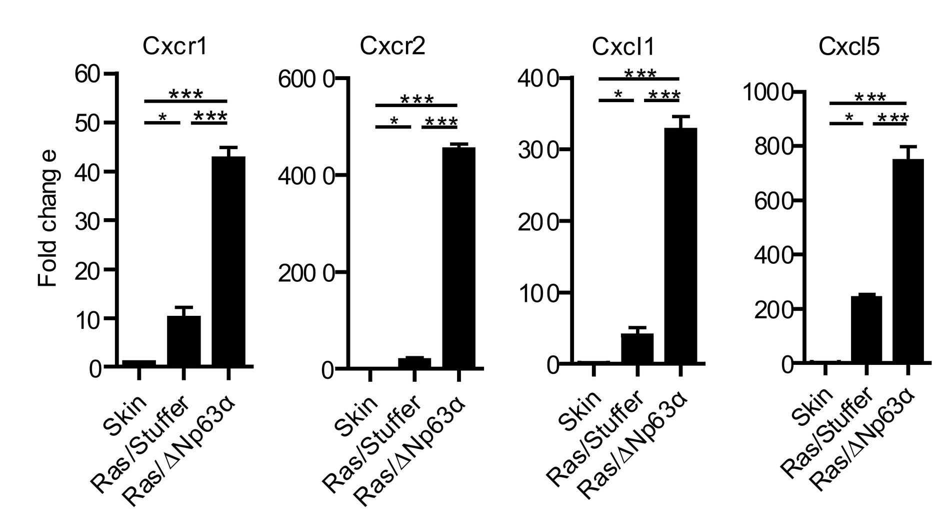


Figure 3. Chemokines and receptors involved in enriching immunosuppressive TME are upregulated in malignant tumors. RT[2] PCR custom array was performed using the RNA samples harvested from tumors. The gene expression of chemokines and receptors known to be responsible for MDSC (top panel) and Treg (lower panel) trafficking is shown.

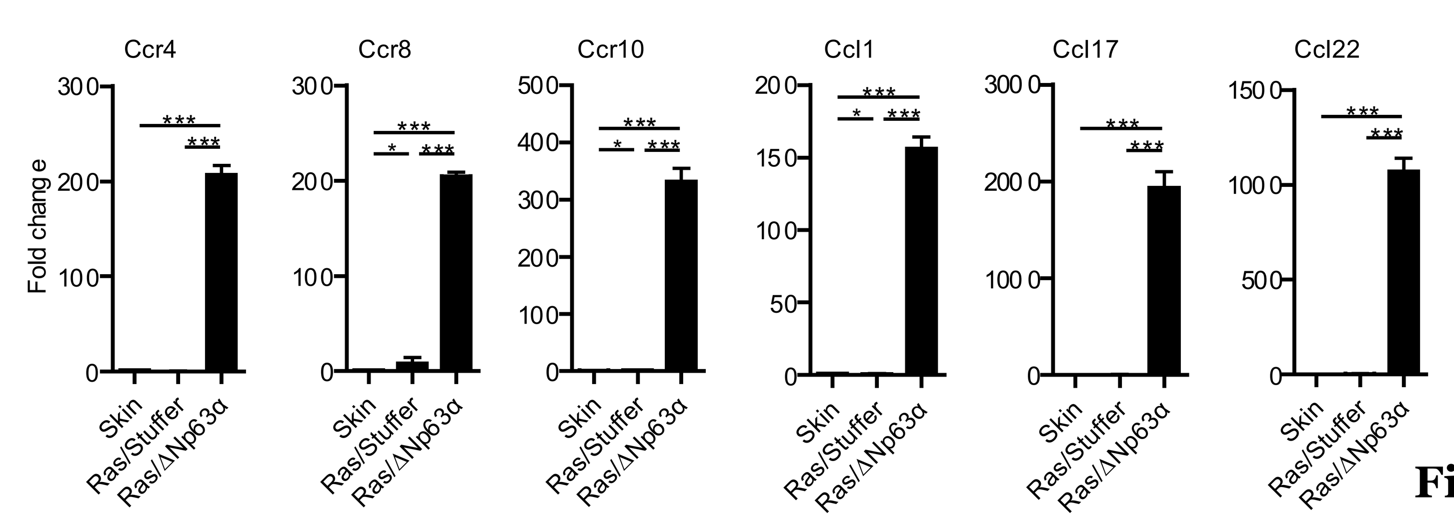


Figure 3.

Discussion & Conclusion

- MDSCs are found at very low levels in healthy individuals but are elevated in obese/diabetic or pregnant populations. They are also elevated in cancer patients and promote pro-tumor microenvironment by suppressing cytotoxic T cell/NK cell activity and promoting angiogenesis.
- This model of multi-step squamous cell carcinogenesis reiterates molecular changes seen in human HNSCC (Activated Ras and ΔNp63α).
- Increased numbers of PMN-MDSCs are recruited to malignant (Ras/ΔNp63 expressing) tumors vs. benign (Ras) tumors in association with increased expression of chemokines involved in MDSC recruitment.
- These findings suggest that Ras/ΔNp63 cooperate to enrich the immunosuppressive tumor microenvironment.
- This study will be extended to evaluate the impact of specific immune cells on clinical efficacy of mAb therapies