Editorial

Epigenetic profiling of tumor infiltrating lymphocytes

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Blocking T cell inhibitory signals, or "checkpoint blockade", has provided unprecedented clinical results for therapy of cancer (1). Antibodies that block negative interactions between programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) allow activation of T cells that recognize tumor antigens and promote immune destruction of the tumor. To determine the activities of T cells of tumor infiltrating lymphocytes (TILs), and to determine which patients will most effectively respond to checkpoint blockade therapies, Schietinger and colleagues developed a novel inducible autochthonous tumor in AST × CreER^{T2} mice. In this system, they successfully analyzed TIL activity and chromatin remodeling during tumor development (2,3).

Chromatin state correlates with rescue of tumor infiltrating CD8+ T cell function

As a model for human hepatocellular carcinoma (HCC), Schietinger *et al.* engineered liver-specific expression of SV40 large T antigen (TAg) to initiate malignant transformation (2). In this tumor, TAg was both the tumor-specific antigen and the tumor driver. Using a lox-stop cassette, expression of TAg was activated when the mice were placed on tamoxifen. They reported morphologic alterations in the liver within 8–10 days, early pre-malignant tumors by 30 days, and frank tumors within 3–4 months (4). All mice succumb to HCC by 4–5 months. Thus, although the AST × CreER^{T2} tumors are initiated in response to one of the strongest oncogenes known, the tumor develops *in vivo*, a benefit lost in transplantable tumors used in many pre-clinical tumor immunology experiments.

To determine whether tumor-specific T cell dysfunction is reversible, monoclonal T cells from TCR transgenic mice specific the SV40-I epitope (5) were transferred into the AST × CreER^{T2} mice. Tumor-specific T cells were synchronized with respect to when they entered the tumor and were easily distinguished from the endogenous repertoire by a congenic marker. Naïve transgenic CD8+ T cells were injected into mice one day prior to inducing TAg expression with tamoxifen, so the T cells would differentiate as the tumor developed. The authors showed that T cell dysfunction was reversible until day 8 and by day 30 T cells could no longer be rescued by transfer into an antigen-free wild-type host or treatment with PD-1 blockade (2).

Next Schietinger's group tested the hypothesis that this irreversible state of dysfunction is epigenetically imprinted in an elegant follow-up manuscript published in *Nature* (3). Epigenetic events are chromatin-based modifications that regulate DNA-templated processes such as transcription, DNA repair, and cellular replication. For decades now, the role of epigenetics in cancer has been investigated with the goal of identifying targets for therapies. The excitement in understanding how epigenetics plays a part in cancer biology has been renewed with the development of new techniques that incorporate next generation sequencing (6). Original methods to investigate epigenetic characteristics were only compatible with large numbers of homogeneous cells (typically cultured *in vitro*), but new technologies facilitate analysis of samples containing few primary cells (7), such as primary T cells found in the tumor microenvironment. Despite studies that have defined molecular programming of T cell activity (8-13), little is known regarding the chromatin and transcriptional dynamics that program TIL activity.

Using the powerful combination of epigenetic and transcriptional profiling to accurately identify the molecular

programming defining T cell function, Schietinger's group showed temporal changes in the chromatin state that define tumor-specific T cell dysfunction (3). Using the Assay for Transposase-Accessible Chromatin (ATAC-seq) in combination with RNA-sequencing (RNA-seq), they showed that tumor-specific T cells differentiated through two distinct chromatin states that correlate with the reversibility identified in their first manuscript (2). The earlier time points (day 7 and before) corresponded to a more plastic reversible state, and later time points (day 14 and after) corresponded to a fixed irreversible state. Notably, when comparing T cells in the tumor relative to T cells that were activated by *Listeria monocytogenes* expressing TAg, the enhancer peaks in the *Ifng* locus that were normally open during effector differentiation were inaccessible when the T cells were dysfunctional. Consistently, negative regulators such as *Ctla4*, *Pdcd1*, and *Tigit* had increased accessibility. The NFAT transcription factor family members C1 and C2, which regulate T cell function, were also accessible in the TILs.

To distinguish readily identifiable proteins that associate with the plastic or the fixed chromatin state in TILs, RNA sequencing was analyzed for cell surface proteins and confirmed by flow cytometry. CD38, CD101, and CD30L expression were up-regulated in the fixed T cells, and CD5 was up-regulated in the plastic cells. Intergenic and intronic peaks in the CD38 locus were uniquely accessible in the fixed state. The authors suggest that these markers may help identify patients who will respond to PD1 therapy as more plastic T cells are amenable to reprogramming before they convert to a dysfunctional state.

AST × CreE^{RT2} *vs.* B16-Ova study

Another group recently reported analyses of tumor-specific T cells from another tumor model using similar techniques (14). Mognol and colleagues performed ATAC- and RNA-seq on Ova-specific TILs from the transplantable B16-Ova melanoma. In these experiments, CD8+ Ova-specific TILs from OT-1 transgenic mice were stimulated in culture and injected into wild-type mice bearing established B16-Ova tumors. Eight days later, TILs were isolated and analyzed. The control in these experiments were transferred tumor nonreactive T cells from P14 transgenic mice. This study was designed to understand exhaustion and focused on finding NFAT and Nr4a as key transcription factors.

A number of similarities and differences were observed from results of both the AST × CreER^{T2} and B16-Ova experiments. Both studies analyzed TILs with an exhausted molecular signature (PD-1+, Tim-3+) and dysfunctional phenotypes were observed only in the tumor-reactive T cells. Specifically, both systems find expression of exhaustion genes on the tumor antigen-specific T cells, but not the irrelevant transferred T cells: OT-1 T cells were not exhausted in the AST × CreER^{T2} tumors (2), and P14 T cells were not exhausted in the B16-Ova tumors (14). In addition, anti-PD1/PD-L1 treatment led to reversal of dysfunction in both systems, depending on the conditions. Lastly, the role of NFAT transcription factor family members were revealed as an important promoter of T cell exhaustion. However, likely due to the differences in the tumor models and experimental methods from each study (*Table 1*), we found little overlap, just 5 genes overlapped between the RNA-seq and ATAC-seq datasets (*Figure 1*). The disparity between these two studies emphasizes the importance of carefully choosing the model and experimental methods to address the hypotheses in question.

Summary

Schietinger's group has developed an inducible autochthonous tumor model, the AST × CreER^{T2} model, which faithfully represents the pathogenesis of cancer. Genetic initiation of tumorigenesis and transfer of naïve T cell prior to tumorigenesis avoids the inflammation associated with transfer of large numbers of tumor cells (17) and allows *in situ* differentiation of cytotoxic T cells, respectively. Using the inducible SV40 model, Schietinger and colleagues have confirmed the power of combined chromatin and transcriptional profiling to accurately identify the molecular programing defining cancer-associated immune cell function. Their recent *Nature* paper convincingly shows two discrete chromatin states in TILs: a more plastic-reversible state, which they propose reflects TILs that can respond to checkpoint blockade therapies, and a fixed state, which is not reversible and is not expected to respond to such therapies. We eagerly await future publications suggesting whether the cell-surface markers identified in this study will distinguish these states and whether they predict responses to checkpoint blockade therapies in human cancer patients. The disparities observed in the molecular programming of TIL function between the different scientific groups may reflect differences in TIL programming in diverse human tumor microenvironments. Thus, we expect that studies designed to further our understanding of the programs that dictate tumor-type-specific T cell dysfunction will point to new targets and strategies that transform dysfunctional T cells into mediators of potent antitumor immunity in diverse tumor microenvironment settings.

Table 1 Differences in the tumor models and experimental methods between Schietinger's manuscripts [AST × CreER^{T2} (2,3)] and Mognol *et al.* [B16-Ova (14)]

Model	AST × CreE^{RT2}	B16-Ova
Tumor	Mice transgenic for tamoxifen-inducible SV40 large T antigen (TAg) expressed to initiate transformation in liver cells	Transplantable melanoma engineered to express the exogenous antigen chicken ovalbumin
Adoptively transferred transgenic T cells	Sorted naïve transgenic T cells specific for the SV40-I epitope were transferred 1 day prior to induction of TAg expression	Transgenic T cells specific for chicken ovalbumin (OT-1) were stimulated with CD3 and CD28 antibodies prior to transfer. T cells were transferred 8–10 days after the tumor
Origin of transgenic TCR	The T cell clone, specific for the H-2D^{b}-restricted SV40-I epitope (SAINNYAQKL), was identified from spleen cells of mice immunized with TAg and stimulated in culture (15)	The OT-1 transgenic TCR, specific for the H-2K^{b}-restricted chicken ovalbumin epitope (SIINFEKL), was identified from the 149.42 T cell clone (16)
Day of epigenetic T cell analysis	SV40-I TILs were analyzed 5, 7 or 14+ days after induction of TAg expression in liver cells	OT-1 TILs were analyzed 8 days after transfer
T cell populations compared	Compared time course of tumor-reactive SV40-I liver T cells to naïve, effector, and memory T cells	Tumor-reactive OT-1 and tumor-ignorant P14 (specific for gp33 from LCMV) TILs

TILs, tumor infiltrating lymphocytes.

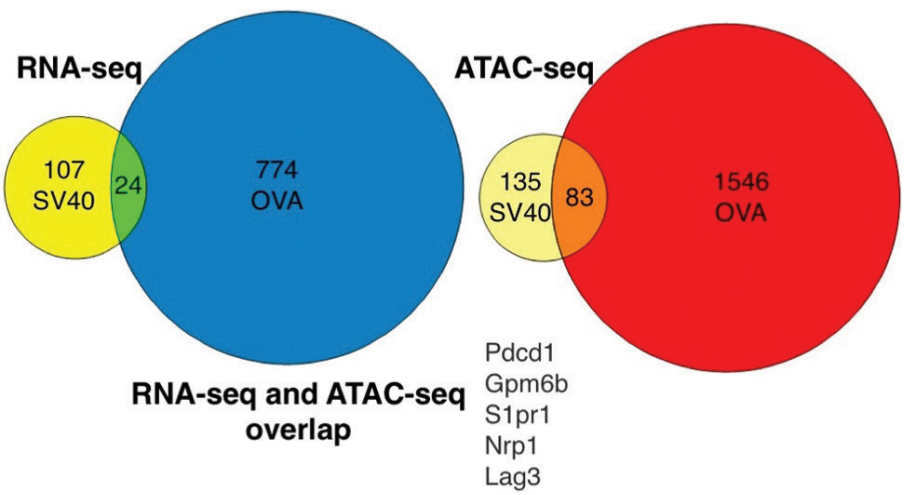

Figure 1 Signatures from PD-1+/Lag-3+ TILs of two recent papers differ. SV40 (3) and Ova-specific (14) TILs were examined by RNA- and ATAC-seq. The list of genes was identified in both systems by both RNA- and ATAC-seq. RNA-seq analysis: The data sets of the 50 most differentially expressed genes encoding membrane proteins in state 1 (L5 and L7) *vs.* state 2 (L14–60) [Figure 3C, (3)] and top 100 most differentially expressed TCR signaling genes during the L7 to L14 transition [Extended Data Figure 6D, (3)] were compared to the differentially expressed genes 8 days after transfer into tumor-bearing recipients [supporting information, dataset S01, OT-1 up and P14 up, (14)]. ATAC-seq analysis: The data set of gene-associated peaks [Extended Data Figure 5 Sector 1 (up in L5/N, but not significant in E5/N) and sector 2 (down in L5/N, but not significant in E5/N) (3)] were compared to both genes associated by proximity to regions and accessibility [dataset S03 OT-1 *vs.* P14 (14)]. The ATAC-seq peaks were within the authors published cut offs: within 2 kb (3) and 200 kb (14) of the genes. Gene sets were compared using the VIB/University of Ghent Bioinformatics and Evolutionary Genomics Web Tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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