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Lung Cancer Subtypes Generate Unique Immune Responses

Stephanie E. Busch,* Mark L. Hanke,* Julia Kargl,†* Heather E. Metz,* David MacPherson,‡ and A. McGarry Houghton*†‡§

Lung cancer, the leading cause of cancer-related deaths worldwide, is a heterogeneous disease comprising multiple histologic subtypes that harbor disparate mutational profiles. Immune-based therapies have shown initial promise in the treatment of lung cancer patients but are limited by low overall response rates. We sought to determine whether the host immune response to lung cancer is dictated, at least in part, by histologic and genetic differences, because such correlations would have important clinical ramifications. Using mouse models of lung cancer, we show that small cell lung cancer (SCLC) and lung adenocarcinoma (ADCA) exhibit unique immune cell composition of the tumor microenvironment. The total leukocyte content was markedly reduced in SCLC compared with lung ADCA, which was validated in human lung cancer specimens. We further identified key differences in immune cell content using three models of ADCA driven by mutations in Kras, p53, and Egfr. Although Egfr-mutant cancers displayed robust myeloid cell recruitment, they failed to mount a CD8+ immune response. In contrast, Kras-mutant tumors displayed significant expansion of multiple immune cell types, including CD8+ cells, regulatory T cells, IL-17A–producing lymphocytes, and myeloid cells. A human tissue microarray annotated for Kras and Egfr mutations validated the finding of reduced CD8+ content in human lung ADCA. Taken together, these findings establish a strong foundational knowledge of the immune cell contexture of lung ADCA and SCLC and suggest that molecular and histological traits shape the host immune response to cancer. The Journal of Immunology, 2016, 197: 4493–4503.

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Address correspondence and reprint requests to Dr. A. McGarry Houghton, Clinical Research Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North (D4-100), Seattle, WA 98109. E-mail address: houghton@fhcrc.org

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Abbreviations used in this article: ADCA, adenocarcinoma; AdCre, adenoviral Cre recombinase; BALF, bronchoalveolar lavage fluid; FVD, Fixable Viability Dye; GEM, genetically engineered mouse; HBC, immune checkpoint inhibitor; IC, circulating immune cell; IC, immune checkpoint inhibitor; IFN, interferon; IL, interleukin; Kras, Kirsten RAS; LCA, lymphoid; LDCS, lung adenocarcinoma; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NKG2D, NK group 2, member D receptor; NSCLC, non–small cell lung cancer; SCLC, small cell lung cancer; TCA, tumor-associated; TCR, T-cell receptor; TME, tumor microenvironment; Treg, regulatory T cell; wt, wild-type.

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Materials and Methods

Mice
All animal experiments used aged-matched mice and were conducted at the Fred Hutchinson Cancer Research Center using protocols approved by the Institutional Animal Care and Use Committee. TetO-EgfR/Cre knockin mice (24) were obtained from the Mouse Models of Human Cancer Consortium on a C57BL/6 background. Cspc-rtTA mice (25) on an FVB background were provided by Jeff Wiester (University of Cincinnati, Cincinnati, OH). Mice were washed, stained with trypan blue, fixed, and permeabilized with a 0.1% Triton X-100 solution. Cell viability was determined using trypan blue staining and a TC20 Auto-count. Cells were washed, stained with FVD, and counted in a hemocytometer. Cspc-rtTA mice were mixed C57BL/6 × 129 background. EGFR and control mouse lung lobes (Cspc-rtTA or TetO-EgfR/Cre) were fed food impregnated with 200 mg/kg doxycycline (Harlan, Indianapolis, IN).

Flow cytometry
Single-cell suspensions were generated from saline-perfused mouse lungs using mechanical disruption, followed by a 1-h digestion at 37°C in RPMI 1640 containing 10% FCS and penicillin/streptomycin, 80 U/ml DNase, 300 U/ml collagenase Type 1 (both from Worthington Biochemical, Lake-wood, NJ), and 60 U/ml hyaluronidase (Sigma, St Louis, MO). D digested lungs were sheared through a 19-gauge needle, strained through 70-μm nylon mesh, centrifuged, lysed (RBCs), washed, strained through 40-μm mesh, centrifuged, and resuspended in Dulbecco’s PBS + 2% FCS. Cell viability was determined using trypan blue staining and a TC20 Automated Cell Counter (Bio-Rad, Hercules, CA). We obtained two SLC2 and five adCA surgical specimens, each with nonadjacent normal lung tissue, using the approved Institutional Review Board file number 6663 in association with Fred Hutchinson Cancer Research Center, University of Washington Medical Center, and NorthWest BioTrust. Single-cell suspensions were generated using the above digestion protocol.

Flow cytometry
Single-cell suspensions were incubated with 1.0 μg of Mouse TruStain FeX and 1.0 μl of Human TruStain FeX (both from BioLegend, San Diego, CA) per 10^6 cells prior to immunostaining. Twenty-seven fluorochrome-labeled Abs were distributed among four multicolor panels for mouse specimens (all flow Abs are detailed in Supplemental Table I). Immunostaining was performed for 30 min on ice, protected from light. Dead cells were excluded with FlxR viability dye (FVD) eFluor 780 (Bioscience), per the manufacturer’s protocol. Stained cells were washed, fixed with IC Fixation Buffer (eBioscence), and stored at −4°C until analysis.

Intracellular cytokine production was assessed using PMA (25 ng/ml), ionomycin (1 μg/ml; both from Sigma), and monensin (1.5 μl/ml; BD Bioscience) stimulation for 5 h at 37°C, 5% CO2. An unstained sample was incubated in the absence of PMA and ionomycin. After stimulation, cells were washed, stained with FVD, fixed, and permeabilized with a Transcription Factor Buffer Set (BD) prior to immunostaining.

Samples were analyzed on an LSR II flow cytometer with FACSDiva software (BD), and ≥1 × 10^6 events were recorded per sample. Data were compensated and analyzed with FlowJo software (TreeStar, Ashland, OR). Gates were defined by fluorescence-minus-one samples and verified with appropriate isotype controls. The unstained control was used to define cytokine gates. Total cell content was calculated by multiplying the overall number of live cells recovered from each animal (i.e., the trypan blue–negative hemocytometer count) by the percentage of live cells for each gated parameter. Cytokine-producing T cell subsets were calculated by multiplying the percent parent gate with the previously determined parent population count. The median fluorescence intensity (MedFlI) of NK cell, CD8+ T cell, and CD4+ T cell populations was calculated in FlowJo, and Med.F.I. of the relevant fluorescence-minus-one control was subtracted from all experimental values for normalization.

CFSE assay
Splenocytes from wt mice were labeled with 50 μM CFSE (Molecular Probes, Eugene, OR), per the manufacturer’s instructions. A total of 1 × 10^6 CFSE-labeled splenocytes was transferred to six-well tissue culture plates coated with anti-CD3/anti-CD28 Abs (BioLegend). Cells were incubated with 200 μg of homogenate, generated from 10-wk Egfr, Kra5, or wt lungs, at 37°C, 5% CO2 for 4 d. Cytokine production was determined by harvesting the cells, staining with CD69-PerCP-Cy5.5 (BioLegend) and FVD, and measuring ≥1 × 10^6 live CD8+ cells on an LSR II flow cytometer.

Gene expression analysis
Total RNA was isolated from frozen mouse lungs using TRIzol reagent (Life Technologies, Carlsbad, CA) and subsequently purified with an RNaseasy Mini Kit (QIAGEN, Hilden, Germany). CDNA was generated from 2 μg of total RNA using SuperScript II Reverse Transcriptase and oligo(dT) (Life Technologies). The expression of indicator target genes was analyzed using a StepOnePlus Real-Time PCR System and TaqMan primer/probe sets (Applied Biosystems, Foster City, CA), with all reactions run in triplicate. Δ Cycle threshold values were calculated using Gapdh as the endogenous housekeeping gene.

Tissue microarray
A lung ADCA cohort on a tissue microarray (TMA), consisting of 135 cases, was obtained from the University of Pittsburgh Cancer Institute. Patient identifiers were removed; therefore, the study was considered not human subjects research (i.e., Institutional Review Board exempt). Each case was previously annotated as EGFR mutant (n = 31), KRAS mutant (n = 9), or wt for EGFR and KRAS (n = 35). Formalin-fixed paraffin-embedded sections were stained with an anti-human CD8 Ab (catalog number ab4055; Abcam, Cambridge, MA). Immunohistochemical (IHC)-stained TMA slides were scanned in brightfield with a 20× objective using a NanoZoomer Digital Pathology System (Hamamatsu, Hamamatsu City, Japan). Twenty TMA cases (n = 5 EGFR and 16 KRAS) were excluded as lost, noninformative (e.g., poorly stained or nontumorous), or exhibiting high interpunch variability (i.e., SEM ≥ 50% of mean). The number of CD8+ cells per TMA core was recorded blind to genotype and normalized to core area. Individual core counts from two or more replicates were available for most cases, and CD8+ cell counts per square millimeter were averaged across replicates. Cut-off values of low versus high CD8+ cell content were defined by the midpoint. Comparison of TMA cohorts was conducted using the Fisher exact test with a one-tailed p value.

Statistical analysis
Significant differences between experimental groups were determined in Prism 6 (GraphPad, La Jolla, CA) using unpaired t tests or, for comparing at least three groups, one-way ANOVA with the indicated post hoc test for correction of multiple comparisons. If the incidence of a particular genotype in the incident group was 0%, the mean ± SEM of the percentage of live cells per lobe was compared between genotypes using the χ^2 test. Unless indicated otherwise, data are presented as mean ± SEM. The p values < 0.05 were considered statistically significant.
**Results**

Egfr- and Kras-driven ADCAs induce a strong inflammatory response

Lung tumor development and associated inflammation were assessed in Egfr, Kras, and Kp53 mice. To allow for the dynamic assessment of TA immune responses, lung tumor–bearing animals and appropriate littermate controls were studied for 6, 10, and 14 wk post-initiation. Consistent with previous studies (24, 26, 34), Egfr, Kras, and Kp53 mice developed neoplastic lesions reminiscent of human disease, from benign hyperplasia and adenomas to malignant ADCAs (Fig. 1A–C). Hyperplasia was observed at all time points, although it was less prevalent in Egfr mice than in Kras-mutant mice (data not shown). The introduction of a secondary mutation in Trp53 increased ADCA formation and amplified tumor growth. Accordingly, tumor burden in 10-wk Kras mice was significantly less than observed in age-matched Kp53 mice (Fig. 1D, p = 0.0231). Analysis of Kp53 mice at the 14-wk time point was precluded by early mortality, but Kras mice remained viable and exhibited 38% lung tumor burden. Body mass measurements, used to noninvasively monitor lung TA morbidity, correlated with tumor burden for all genotypes (Fig. 1E).

Previous investigations of pulmonary inflammation were largely based on the assessment of bronchoalveolar lavage fluid (BALF). Although a well-accepted methodology, BALF studies confine analysis to the airway compartment and limit the number of immune cell types that can be identified. Therefore, to more thoroughly investigate the immune cell composition of the TME, we performed flow cytometric analyses on single-cell suspensions generated from whole-lung tissues. Using the gating strategy shown in Fig. 2A, we identified 13 unique leukocyte populations defined by 16 Ab markers (Materials and Methods, Supplemental Table I), Kras-, Kp53-, and Egfr-mutant mice display a robust immune response, as evidenced by 3-5-fold increases in total CD45+ cell content compared with normal lung (Fig. 2B-G).

Macrophages were by far the most prevalent immune cell type in the lungs of the three murine ADCA models. Ten weeks after starting doxycycline, Egfr mice exhibited 11-fold increases in macrophage content compared with controls (Fig. 2B). Similarly, by 6 and 10 wk post-Cre induction in Kp53 and Kras animals, respectively, total macrophage cell counts had increased 14- and 17-fold (Fig. 2D, 2F). Of note, macrophage content increased with time only in Kras and Kp53 animals (Supplemental Table II). Because myeloid-derived suppressor cells (MDSC) are composed of monocytic MDSC and granulocytic MDSC subsets, we simply defined these cells as monococytes (CD11b+Ly6C+) and neutrophils (CD11b+Ly6G+). We observed small, but significant, differences in neutrophil content in 10- and 14-wk Egfr tumor-bearing lungs (Fig. 2B, 2C), as well as in 14-wk Kras tumor-bearing lungs (Fig. 2E). By 6 and 10 wk, TA neutrophil content increased 2- and 5-fold, respectively, in Kp53 animals compared with control (Fig. 2F, 2G). Indeed, neutrophil counts in Kp53 lungs were statistically significantly increased compared with all other genotypes at the 10-wk time point (Supplemental Table III). However, this neutrophil signature may merely reflect overall tumor burden, because statistical analysis of cohorts with approximately matched tumor area (i.e., 6-wk Kp53, 10-wk Kras, and 14-wk Egfr) failed to identify significant differences in pulmonary polymorphonuclear cell content.

**Impaired NK cell function in Kras-driven ADCA**

NK cells are lymphocytes of the innate immune system that play an important role in the host defense against inhaled pathogens (35). NK cell counts in nontumor-bearing lungs were comparable to those of macrophages and granulocytes (Fig. 2). However, unlike the myeloid cell expansion observed with ADCA development, NK populations remained largely unaltered in tumor-bearing lungs. When significant increases were identified (Fig. 2B, 2D–G), the fold changes were small, and NK cell counts decreased over time in Kras animals (Supplemental Table II). NK cells are required for effective tumor immunosurveillance (36), but cancer cells have developed multiple strategies to escape NK cell–mediated cytotoxicity, including downregulation of NKG2D (also known as CD314) (37, 38). Surface expression of NKG2D on NK cells was decreased significantly in Kras- and Kp53-tumor–bearing animals at all time points but exhibited no change in Egfr mice (Fig. 2H). Thus, these murine models of Kras-driven lung ADCA recapitulate an immune-escape mechanism previously described in human lung cancer (39).

**Oncogenic drivers dictate lymphocyte recruitment into the ADCA microenvironment**

The majority of immunotherapeutic approaches are based on the ability of the adaptive immune system to infiltrate tumors and identify tumor-specific Ags. Therefore, we comprehensively surveyed the lymphocyte subpopulations present within the TME. B cell populations remained unaltered in tumor-bearing Egfr mice (Fig. 2B, 2C) but increased ≥2-fold in Kras and Kp53 mice at all time points (Fig. 2D–G). CD3+ populations were significantly increased in multiple groups (Fig. 2B, 2D–H), but T cell counts were demonstrably greater in Kras and Kp53 mice compared with Egfr mice (Supplemental Table III), even after controlling for tumor burden.

CD4+ Th cell expansion was observed in both Kras-driven tumor models but was limited in Egfr mice (Fig. 3A–F). This pattern was reflected in Treg content, which was significantly lower in tumor-bearing lungs from Egfr mice compared with Kras mice (Supplemental Table III). Interestingly, Treg content increased over time in Kras and Kp53 animals (Supplemental Table II), the only cell type other than macrophages and neutrophils to exhibit such dynamic behavior. Expression of IFN-γ by CD4+ T cells (Th1 cells) and IL-17A by CD3+ T cells was also significantly upregulated in Kras and Kp53 animals compared with control at early and late time points (Fig. 3C–F) but exhibited little to no increase in Egfr animals (Fig. 3A, 3B). Direct comparison of tumor-bearing lungs from all genotypes found higher Th1 and CD3+IL17A+ cell counts in both Kras-driven models compared with Egfr mice (Supplemental Table III). Despite repeated attempts, we were unable to identify IL4+ Th2 cells in our animals (Supplemental Fig. 1); it remains unclear whether this deficiency reflects a true biological absence or, more likely, a technical barrier.

To assess the spatial relationship between lymphocytes and tumor cells, we performed IHC staining for CD3 and Foxp3 on Egfr-, Kras-, and Kp53-tumor–bearing mice. For each model of lung ADCA, immune cell location was identified as TA (or peripheral), TI, or within a neighboring LA. Examples of each are provided in Fig. 3G-J. Staining for CD3 illustrated key differences by genotype, because Egfr-mutant mice displayed markedly fewer TA CD3+ T cells than Kras and Kp53 specimens (Fig. 3K, left panel). TI CD3+ cells were identified in all ADCA models, although they were more prevalent in Kp53 mice (Fig. 3K, middle panel). Similarly, CD3+ cells were present in all LAs, but LAs were significantly less common in Egfr mice but were uniformly present in the other genotypes (Fig. 3K, right panel). Approximately 15% of lobes from Egfr mice contained TA Tregs compared with ~40% for Kras mice (Fig. 3L, left panel). The primary location of Tregs in all genotypes was within LA structures (Fig. 3L, right panel), with essentially no tumor infiltration observed (Fig. 3L, middle panel). Taken together, the IHC studies confirm the flow cytometry data showing that Egfr...
mice contain fewer Tregs than the other genotypes and, moreover, demonstrate a paucity of TI lymphocytes, especially compared with Kp53 tumors.

**CD8⁺ lymphocyte content and function differ by lung ADCA subtype**

CD8⁺ T cells are capable of detecting and discriminately eliminating tumor cells (40). Notably, CD8⁺ cell content differed significantly between models driven by mutant Kras versus Egfr. Expansion of CD8⁺ cells was observed at all time points in Kras and Kp53 mice but did not occur in the Egfr-mutant cohort (Fig. 3A–F). Even after controlling for tumor burden, Kras-mutant mice displayed greater CD8⁺ cell content than did Egfr mice (Supplemental Table III). Therefore, we carried out a number of experiments in an attempt to determine the mechanistic basis for this finding and to translate this observation to human disease.

Initially, we performed quantitative real-time PCR for key CC and CXC chemokines known to impact immune cell recruitment. Notably, we identified an increase in Cxcl-9 and Cxcl-10 in Kras⁻mutant lungs compared with Egfr (Fig. 3M), which may explain,
at least in part, the differences in lymphocyte content seen between the two lung ADCA models.

To translate these findings to human disease, we performed IHC staining for CD8 on a lung ADCA TMA annotated for KRAS ($n = 53$ cases) and EGFR ($n = 26$) mutational status. The frequency of KRAS and EGFR mutations in the cases displaying high versus low CD8 content (the top 50% and bottom 50% of cases, respectively) was assessed, and EGFR mutations were found to be significantly overrepresented in the CD8-low cohort (Fig. 4A). Specifically, 65.4% of EGFR-mutant cases were scored as CD8-low versus 41.5% of KRAS-mutant cases. Thus, similar to the findings in the GEM models presented above, EGFR-mutant lung
ADCAs exhibit reduced CD8+ lymphocyte infiltration in human lung cancers compared with KRAS-mutant lung ADCAs. Because CD8+ responses can be blunted by immune checkpoint ligands, we measured PD-L1 expression on macrophages and tumor cells (EpCAM+) by flow cytometry. Interestingly, although PD-L1 expression was decreased in tumor-bearing versus control lungs for Egrf and Kras mice, there was no difference in PD-L1 expression between oncogenic subtypes (Fig. 4B). Because other tumor microenvironmental factors can perturb lymphocyte function, we assessed whether the TME of Egrf mice was more suppressive to lymphocyte proliferation than that found in Kras mice. Egrf and Kras tumor homogenates reduced CD8+ T cell proliferation using CFSE-labeled lymphocytes, but the Egrf homogenates were not more suppressive than Kras (Fig. 4C).

Because the intriguing lack of CD8+ cell expansion in Egrf-mutant tumors suggests a failure of CD8+ cell activation in Egrf mice, we performed a detailed assessment of T cell effector and memory status at the 10- and 14-wk time points using the markers CD62L, CD44, and PD1 (Fig. 4D). No evidence of CD8+ T cell activation was observed, as reflected by the lack of an increase in CD8+PD1+ cells in tumor-bearing Egrf mice (Fig. 4E, 4G). Additionally, the proportion of central memory (CD62L+CD44+) and effector/effector memory (CD62L−CD44+) CD8+ T cells was unchanged, with the majority of these cells still falling into the naive (CD62L+CD44+) category in Egrf mice. In contrast, and consistent with the small, but significant, increase in Th cells shown in Fig. 3A and 3B, CD4+ T cells demonstrated a significant increase in effector/effector memory populations in 10- and 14-wk Egrf mice (Fig. 4F, 4H).

Given the robust increase in CD8+ T cell content observed in tumor-bearing lungs from Kras mice compared with control, we elected to assess the status and functionality of the lymphocyte populations in Kras mice using a CTLA4 mIgG2b (clone 9D9) Ab (MedImmune). Although administration of anti-CTLA4 increased the proportion of CD8+ (Fig. 4J) and CD4+ (Fig. 4K) effector/effector memory cells, this cellular phenotype failed to translate into an altered tumor burden in Kras mice (Fig. 4I). Thus, despite an activated CD8+ T cell response in Kras-mutant mice, tumor progression continued unabated.

Role of IL-17A–producing γδ T cells in Kras-driven lung ADCAs

Given the robust expansion of IL-17A+ T cells in Kras-mutant tumor-bearing mice and the known protumor role of Th17 cells in...
lungen ADCA (41), we elected to examine the cellular sources of IL-17A in the lungs of our Kras-mutant mouse models. Surprisingly, the predominant source of IL-17A was found to be γδ T cells rather than CD4\(^+\) Th17 cells (Fig. 5A, 5B). An attempt to interrogate the role of IL-17A in lung tumorgenesis by crossing Kras-mutant mice to mice lacking the transcription factor for IL-17A (i.e., ROR\(\gamma\)) (42) was stymied by the frequent occurrence of lymphoid neoplasms in these animals. The spontaneously arising lymphomas exhibited thymic (Fig. 5C) and splenic (Fig. 5D) involvement, as well as diffuse infiltration of the liver (Fig. 5E) and lungs (Fig. 5F). Previous studies of a related mouse model of ROR\(\gamma\)t deficiency (43) similarly identified a high incidence of lymphoma but did not detect pulmonary metastases that, unfortunately, preclude the use of this model in lung tumorgenesis studies. Further efforts to interrogate the specific role of γδ T cells in Kras-mutant lung ADCA revealed that deletion of γδ T cells did not impact tumor burden (Fig. 5G) or the immune cell composition of the TME (Fig. 5H).

A paucity of TI leukocytes in murine and human SCLC

The immune cell composition of the SCLC TME has not been investigated to any extent. Therefore, we profiled the immune content of SCLC using cohorts of Rhp53 mice infected with AdCre. As described previously (44), lung tumors of mainly neuroendocrine histology arose within 40–50 wk of AdCre administration (Fig. 6A). Flow cytometric analysis of SCLC tumor-bearing lungs (gated as shown in Fig. 2) identified a small, but noteworthy, inflammatory presence in Rhp53 mice compared with control (Fig. 6B). The total number of CD45\(^+\) leukocytes was increased 2-fold in SCLC, and TA CD45\(^+\) cells were also visible by IHC staining (Fig. 6C). Unlike Kras- and Egfr-mutant animals (Fig. 1A, 1B), SCLC tumors presented as large discrete foci, and little hyperplasia was observed. CD45\(^+\) cells were consequently clustered at the periphery of the SCLC lesions (Fig. 6D), without the inflammatory field effect frequently observed in the ADCA models. Few TI leukocytes were detected (Fig. 6E).

The major immune component of SCLC was found to be CD3\(^+\) T lymphocytes (Fig. 6B). This population included a 7-fold increase in the number of γδ T cells and a strong trend toward increased CD4\(^+\) Th cells (\(p = 0.0698\)). In marked contrast to the ADCA models, expansion of innate immune cells in SCLC tumor-bearing lungs was minimal, with only a 2-fold increase observed in macrophages and a nonsignificant increase in neutrophils (\(p = 0.0886\)). To further investigate this phenomenon, we compared the ratio of CD3\(^+\) T cells/myeloid cells (macrophages, neutrophils, monocytes, and eosinophils) and found a pronounced lymphocyte-dominated signature in SCLC versus all ADCA models (Fig. 6F). Egfr mice presented the smallest CD3/myeloid ratio and were also significantly different from Kras mice.

As part of an ongoing study of the immune composition of human lung cancer, we obtained two surgical specimens with confirmed small cell pathology. Because resecting SCLC is rarely clinically indicated, these two specimens represented a unique opportunity to measure the immune cell composition present within the SCLC TME. Therefore, we performed flow cytometry analyses on single-cell suspensions generated from these two cases. Similar to the findings in the GEM models, TA inflammation was discernibly lower in SCLC compared with five ADCA specimens; CD45\(^+\) cells accounted for a mere 16.2% of live cells in the SCLC resections compared with 81.9% in ADCA (Fig. 6G).

Discussion

Lung cancer is a heterogeneous disease that can be divided into distinct subtypes based on molecular and cellular characteristics (45). In this study, we tested the hypothesis that these subtypes dictate the inflammatory response to cancer by immune profiling the lung TME in a mouse model of SCLC and in three molecularly distinct models of NSCLC (Fig. 6H). We found that Egfr and Kras mutations give rise to distinct immune responses characterized by differential expansion of B cells, CD8\(^+\) T cells, Tregs, and IL-17A–producing T cell populations. Although loss of Trp53 promoted malignancy, it had minimal effect on immune cell composition within the Kras TME. We further demonstrate that SCLC possesses an overall reduced inflammatory presence compared with NSCLC, and one in which lymphocytes predominate over myeloid lineage cells. Therefore, mutational profile and histological origin actively shape the immune contexture of lung cancer, a finding that may have important clinical ramifications.

The strongest macrophage field responses that occur in mutant Kras- and Egfr-driven mouse ADCA are seldom observed in human lung cancer and represent a potential limitation of these GEM models. In Kras mice, this phenomenon of alveolar macrophages flooding the airspaces was likened to desquamative interstitial pneumonitis, a rare interstitial lung disease with similar pathology (46). Moreover, the conditional mouse models in this study use varied induction methodologies (i.e., AdCre- or doxycycline-regulated transgene expression). Although we cannot exclude potential confounding effects of viral infection or doxycycline consumption on the tumor immune response, we attempted to correct for these variables by using adenovirus- or doxycycline-exposed wt animals for the relevant control cohorts.

Few gene-specific investigations of the mouse lung TME have been conducted, and no comprehensive effort has been made to compare and contrast different molecular and histological models of lung cancer. However, our results validate findings from several earlier studies of lung TA inflammation. We were unsurprised to identify macrophages as the dominant immune cell present in mouse ADCA models given that strong TA macrophage responses were identified in mutant Egfr (21) and Kras (19, 41, 47) mouse lung tumor models. Likewise, as we described in this article, neutrophils were shown to be a modest, but important, component of Kras-driven, but not Egfr-driven, mouse lung ADCA (19, 21, 41). Because the majority of prior data in this regard relied on BALF cell counts, we used flow cytometry to better define the quality of the immune response. Using this methodology, we found that recruitment of lymphoid lineage cells varies greatly among ADCA models, because Egfr\(^{L858R}\) mice exhibited a paucity of B cells, CD8\(^+\) T cells, Tregs, and IL-17A–producing T cells compared with the Kras and Kps53 lung TME.

The most clinically relevant finding in this study is the lack of a CD8\(^+\) lymphocyte response in Egfr-mutant mice and EGFR-mutant human lung ADCA specimens compared with their KRAS-mutant counterparts. Markers of effector/memory status failed to reveal any evidence of CD8\(^+\) T cell activation or differentiation in Egfr mice. This suggests that EGFR mutation may not elicit an Ag-driven immune response. Although the same could be said for mutant KRAS, we were able to demonstrate an increase in activated and effector memory CD8\(^+\) cells in the Kras mouse model. Furthermore, TI lymphocyte populations that specifically target mutant KRAS\(^{G12D}\) were identified recently in colon cancer (48). Despite the presence of activated CD8\(^+\) cells, tumor growth continued in Kras mice, even with the addition of an anti-CTLA4 therapeutic Ab. Our interpretation of this data is that increases in activated CD8\(^+\) cells within the TME in Kras-mutant mice do not impact tumor growth unless they are tumor reactive. Specifically in this case, tumor-derived chemokines, such as Cxcl1-10, are likely to increase the number of TA lymphocytes. Although anti-CTLA4 Ab therapy drove an increase in effector T cells, these cells would not be expected to reduce tumor burden if they did not recognize a TA Ag. It is also possible that anti-CTLA4
FIGURE 4. CD8+ cell content and function correlate with lung ADCA subtype. (A) The number of CD8+ cells per square millimeter was tabulated for each core section present on a TMA of lung ADCA cases annotated for EGFR and KRAS mutational status. Cases were ranked from lowest to highest CD8 content prior to unblinding for genotype. Shown are representative images of EGFR-mutant (left panels) and KRAS-mutant (right panels) ADCA. Scale bar, 100 μm. A total of 65.4% (17/26) of EGFR-mutant cases were scored as CD8-low versus 41.5% (22/53) of KRAS-mutant cases. *p = 0.0392, Fisher exact test. (B) PD-L1 Med.F.I. was assessed on pulmonary EpCAM+ epithelial cells and macrophages from 10-wk Egfr mice (n = 5) and 14-wk Kras mice (n = 4). Expression compared with normal lung (n ≥ 4) is shown (lower panels). (C) Splenocytes from nontumor-bearing wt mice were labeled with CFSE and incubated with protein homogenate generated from wt normal lung (NL) or tumor-bearing lung from 10-wk Kras or Egfr mice or with media alone. The cells were subsequently stained with anti-CD8 and a viability marker and analyzed for CFSE intensity; representative plots are shown. For each genotype (n ≥ 3), the percentage of proliferating CD8+ T cells was determined after normalization to the media control. Statistical differences were assessed by one-way ANOVA with the Tukey posttest. (D) Flow cytometric analysis of T cell function in Egfr mice compared with wt control, (Figure legend continues)
Tregs and IL17A+ T cells have emerged as important cell populations in multiple mouse models of cancer (41, 51, 52), and both cell types exhibited notable patterns of expression or localization in the murine ADCA models. Although TGF-β and IL-6 generate gradients leading independently to Treg or Th17 differentiation, we observed concurrent increases in both populations in Kras- and Kp53-mutant tumor–bearing lungs. Notably, we found that the major source of IL-17A in Kras-mutant ADCA was γδ T cells and not Th17 cells. Because IL-17A deficiency was shown to reduce lung tumor growth (41), and IL-17A–producing γδ T cells are known to promote breast (53) and pancreatic neoplasia (52), these findings suggested to us that expansion of a pulmonary IL-17A–producing γδ T cell subset might overshadow the tumor-surveillance role traditionally ascribed to γδ T cells (54). However, Kras-mutant γδ T cell–deficient mice displayed equivalent lung tumor burden and strikingly similar immune profiles to their γδ T cell–competent counterparts. Therefore, although IL-17A is an important signaling component in the immune landscape of lung ADCA, IL-17A+ γδ T cells appear to contribute little to the process of lung tumorigenesis.

Tregs, in contrast, appear to play a particularly important role in the Kras-mutant lung TME, because they were the only nonmyeloid lineage population to expand over the course of tumor development. Moreover, Tregs display a unique anatomic location in lung ADCA. They are rarely associated with the tumor itself; instead, they are frequently found within LA structures that are believed to function as a local site of Ag presentation and are correlated with good clinical outcomes in NSCLC (55). The presence of Tregs in these structures was recently shown to be detrimental to the generation of an effective immune response in murine lung ADCA (56), highlighting the importance of Treg-targeting strategies for the clinical management of lung cancer patients.

The immune cell composition of SCLC has not been well studied. Our findings in Rbp53 mice point to a less robust, but more lymphocyte-predominant, host immune response to murine SCLC than to ADCA. Moreover, when we analyzed the immune cell content of two human SCLC cases, we identified a strikingly similar immune profile of sparse CD45+ cell content. Although we acknowledge the inherent limitations of n = 2 studies, patients diagnosed with SCLC seldom undergo lung resection (57), making access to such specimens exceedingly rare. Solid tumor malignancies demonstrating the best responses to currentICI therapies are those with high mutational burdens and/or a history of cigarette smoke exposure (e.g., melanoma, head and neck squamous cell carcinoma, and urinary bladder cancer), both traits common to SCLC (2). In light of these correlations, it is tempting to speculate that SCLC patients would exhibit good responses to ICI therapy. However, initial reports suggest that success rates for anti-PD1 therapy in SCLC are, at best, only comparable to NSCLC (58, 59). Our preliminary findings with respect to the immune cell composition in SCLC suggest that the presence of redundant immune-suppressive factors would not be a likely source of treatment failure, which is almost certainly an important concept in NSCLC. These findings point to potentially unique features of the SCLC TME (e.g., matrix protein composition) that require additional study.

gated from single live CD45+CD3+ parent population. Lymphocytes were gated as CD45+CD3+ (i.e., Naive), CD45−CD3+ (Tcm, central memory), and CD45−CD3+ (Temem, effector memory/effector). PD1 expression was assessed on CD8+ T cells only. CD8 + and CD4+ T cell populations were examined at 10 (E and F) and 14 wk (G and H) postinduction of mutant Egrf (n = 6 tumor-bearing lungs and n ≥ 3 controls per group), respectively. (b) Percentage lung tumor area of Kras mice treated with anti-CTLA4 (n = 7) or isotype (n = 6) (right panel) and representative H&E sections (left and middle panel). Scale bar, 500 μm. (J and K) Flow cytometric analysis of CD8+ and CD4+ T cell populations in anti-CTLA4–treated and isotype-treated Kras mice (n = 5 per group). *p < 0.05.
A robust CD45<sup>+</sup> immune response was observed in the ADCA mouse models and human lung ADCA patients. Leukocytes account for nearly 75% of total cellular content in human ADCA, which is an even greater proportion than we identified in mice (~55%). With the exception of the aforementioned exaggerated macrophage responses, the robust and diverse immune landscape observed in GEM models of ADCA approximates that seen in human lung cancers (60). Driving mutations, such as in \textit{Egfr} and \textit{Kras}, substantially impact the TME through the release of bioactive molecules, which is very well reflected in these GEM models. One potential shortcoming of these models is the genetic simplicity of the tumors, which rely on a single driving mutation. In contrast, human NSCLC harbors an average of ~150 distinct mutations per case (61). Efforts are underway to construct mouse models of cancer that harbor a greater abundance of single nucleotide variations and, thus, potential neoantigens. However, \textit{EGFR}–mutant cancers in nonsmokers are typically genetically simplified (5), such that the \textit{Egfr}–mutant mice described in this article likely constitute an excellent representation of the genetic component of the cancer cell and the immune composition of the TME.

The emergence of ICIs has been a tremendous advance; unfortunately, the majority of lung cancer patients in clinical trials failed to respond to ICI therapy (8–11). In addition to the PD-1/PD-L1–based drugs currently in use, novel ICI agents are likely to emerge in the near future. Our findings argue that the cellular and molecular characteristics of lung cancer may provide an important framework for patient-targeted immunotherapy. Furthermore, preclinical testing of future immunotherapy agents should be performed in genetically and histologically diverse model systems to enable the assessment of tumor subtype–specific efficacy.

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