INTRODUCTION

The immune system is regulated by activating pathways and inhibitory checkpoints that define the balance between immune protection and tolerance (1–3). Tumor cells can induce checkpoints to evade immune destruction. For example, expression of PD-L1 on cancer cells interacts with PD1 on T cells to inhibit their activation and targeting of cancer cells (4–6). Immunoresponse aimed at blocking this PD-L1-PD1 axis is effective in targeting tumors that are refractory to other treatment protocols, including metastatic melanoma and advanced non–small cell lung cancer (7–11). However, only a subset of patients respond to this immunotherapy, and responders can acquire resistance (9, 10, 12). Expanding the effectiveness of immunotherapy will be facilitated by understanding why most patients do not respond. Beyond inhibition by the PD-L1-PD1 axis, T cells also face a set of immunosuppressive cytokines secreted by alternatively polarized M2-like tumor-associated macrophages (TAMs) that inhibit their migration and activation (13–15). Such a second T cell barrier is thought to be an important component in resistance to checkpoint immunotherapy. Studies are ongoing to understand how some TAMs become alternatively polarized M2 phenotype in tumors. There is also linkage between epithelial mesenchymal transition (EMT) status of solid tumors and their response to immunotherapy and chemotherapy (16–18). EMT is driven by transcription factors that repress epithelial specification genes and induce mesenchymal genes in a transforming growth factor–β1 (Tgf-β1)–dependent fashion, and key to this process in cancer is Zeb1, which drives tumor cell invasion (16, 19–22). Consistently, TGF-β1 signaling, which induces EMT, is likewise linked to therapy resistance (23–26). EMT has been shown to induce expression of PD1 on cancer cell lines, and high levels of PD1 are linked to immunotherapy resistance, implying a potential mechanism for EMT in immunotherapy resistance (18, 27). Because EMT is a driver of cancer cell invasion, its impact in the context of the tumor on immunotherapy would appear to be focused at the cancer stage where invading cells appear. Yet, the notion that invading cancer cells are the target or a regulatory node in a tumor’s response to immune checkpoints is not established, nor is it clear whether invading cancer cells undergoing EMT might be linked to alternative M2 polarization of TAMs.

In a K-Ras model of mouse lung adenocarcinoma (AC), we provide evidence that induction of Zeb1 within a TGFb1-rich environment in invading cells links both of these seemingly disparate checkpoint immunotherapy resistance pathways to tumor invasion. We show that PD1 is confined to invading AC cells, and using Zeb1 mutation, knockdown, and overexpression, we show that Zeb1 is required for this expression of PD1. In addition, we demonstrate that Zeb1 induces CD47 on the invading cells. CD47 can bind Sirpa and Tsp1 on macrophages to trigger a Shp2-dependent pathway that regulates phagocytosis (28). However, here, we show that this Zeb1 induction of CD47 also drives M2 polarization of adjacent macrophages, highlighting a pathway for TAM M2 polarization in tumors, confined specifically to the microenvironment around invading cells. With this PD1 induction and CD47-dependent M2 polarization of TAMs in the microenvironment around invading cells, there is a loss of PD1+ CD8 cells in this region.

Our findings point to invading cells as a key target of immune checkpoint regulation. Zeb1 induction of PD1 and CD47 checkpoints establishes an immune desert surrounding these invading cells, protecting them as they migrate into an inflammatory environment surrounding the tumor.
RESULTS
Zeb1 induction is linked to EMT in invading cancer cells in K-Ras–initiated lung tumors

Mutations in the Ras signaling pathway in mice have been used as models of human lung AC (29). Mutations in K-Ras and epidermal growth factor receptor do not overlap in lung AC, demonstrating that they provide similar stimulation of the pathway in lung cancer (30). Complementary mutations in genes such as p53 and Pten have been used to evaluate their effect on tumor progression in this K-Ras model. Pten is not mutated in lung or pancreatic cancers where K-Ras is mutated, suggesting that this mutation is substituting for repression of Pten that occurs in these tumors (31–33). p53 mutation did not promote tumor initiation, but it did drive a metastatic phenotype (34, 35). Although such compound mutations in mice are informative, it is of note in patients accumulate over a protracted period in patients. Although mouse tumors initiated with the only K-Ras mutation progress more slowly, they might more closely reflect tumor initiation in patients (36–38).

Initially, atypical adenomatous hyperplasia (AAH) originates in bronchial airways, and AAH then by postnatal day 120 (P120) forms lung adenosas (AD) (Fig. 1, A and B) (36, 38, 39). Studies directed at identifying the cells of origin for these tumors have identified both Clara and ATII cell progenitors (39–41). Zeb1 is critical for tumor invasion (42–45), and at P220, it is induced on AC cells at the tumor edge as they begin to invade surrounding lung parenchyma, alveolar space, and large airways, and consistent with EMT driven by this Zeb1 induction, the epithelial specification factor E-cadherin (Cdh1) is repressed and the mesenchymal marker Vimentin (Vim) is induced compared to Zeb1lo AC cells in the central tumor (Fig. 1, C to H). Regions of AD at the lesion edge remain Zeb1-invasive and noninvasive at this age. Human lung AC does not transition through an obvious AD stage, but like the mouse model, Zeb1 is induced on invading AC cells at the tumor edge compared to AC cells in the central tumor (37). In addition, there is a linear relationship between Zeb1 and Vim mRNA levels in human non–small cell lung cancer (Fig. 1I).

Cyclical Zeb1 induction marks transition from cancer cell generation to invasion

Zeb1 is critical for transition of AD cells to AC in this mouse K-Ras model (37, 38). Reducing Zeb1 expression by short hairpin RNA (shRNA) knockdown or heterozygous gene mutation did not affect AD formation and expansion, but AC generation, EMT, and invasion at the tumor edge are inhibited, and accordingly, AD size is similar in wild-type (WT) and Zeb1 mutant lungs until P220, when further WT lesion expansion begins to coincide with invasion of forming AC into surrounding normal lung tissue (Fig. 1, J to M). By P150, small foci of AC, distinguished from surrounding AD cells by their nuclear heterochromatin staining, EMT (loss of E-cadherin expression) and Zeb1 induction begin to appear in the hypoxic centers

![Fig. 1. Zeb1, EMT, and invasion in K-Ras–initiated lung cancer.](http://advances.sciencemag.org/)
of expanding AD (Fig. 2, A to G) (38). As forming AC cells migrate away from these foci, they down-regulate Zeb1 and expand into papillary tumors that obscure the original foci in the central lesion (Figs. 1C and 2, D to G). Mutation or shRNA knockdown of Zeb1 in the lung blocks this foci formation and prevents AC cell generation (Fig. 1M) (38). As AC cells extend from the central tumor to portions of the tumor edge, they reinduce Zeb1 as clusters of the cells begin to invade surrounding lung parenchyma, alveolar space, and large airways (Fig. 1, D to H). AC formation and progression to invasion is then characterized by cyclical Zeb1 levels, with induction evident in small foci of AC generation, down-regulation in expanding AC in the central tumor, and reinduction highlighting AC cells at the invasive front.

**Tgfb1 accumulates at sites of Zeb1 induction in AC**

TGFβ1 is known to induce Zeb1 and drive EMT, and it accumulated specifically around foci of initial AC generation, where Zeb1 was induced (Fig. 2, H to H‴) (38). As AC cells migrate away from these hypoxic, Tgfb1-rich foci down-regulated Zeb1 (Fig. 2, D to H‴). In addition, AC cell expansion in the central tumor continued in the absence of Tgfb1, and the cells maintained Zeb1lo expression (Fig. 1, F and G). However, Tgfb1 also accumulated at regions of the tumor edge closely associated with initial invading AC cells clusters that had reinduced Zeb1 (Fig. 2l). These results imply that accumulation of Tgfb1, a known driver of Zeb1 expression, is responsible for the cyclical induction of Zeb1 in distinct AC microenvironments in expanding lesions.

**A gradient influx of proinflammatory cells characterizes AD**

Even at P220 where invasive AC is evident, lesions remained an intertwined heterogeneous mixture of microenvironments including regions of Zeb1-AD cells, Zeb1lo AC cells in the central tumor in TGFβ1-deficient regions, and clusters of Zeb1hi invading AC cells at the tumor edge at sties of Tgfb1 accumulation. Notably, invading AC cells only comprised a portion of the tumor edge, and they extended from regions of AC in the central tumor. Other portions of the tumor edge still consisted of noninvading AD cells at this age. Identification of the boundaries of these intertwined tumor microenvironments is guided by and dependent upon histology. Attempts at quantification of immune cell populations simply in peripheral versus central tumor regions by flow cytometry leads to a mixture of tumor microenvironments. In addition, immune cell influx into normal lung is also not uniform, as it is concentrated at sites where large airways and adjacent complementary blood vessels are evident, most easily identified by histology. Therefore, we relied upon counting cells following immunostaining for markers in tissue sections where microenvironment boundaries can be clearly defined.

A CD45+ inflammatory infiltrate composed largely of CD8 T cells and M1 polarized macrophages is evident in the uninvolved lung in response to AAH and AD outgrowth (Fig. 1A and fig. S1). However, as noted above, this infiltrate is not uniform in the lung and is concentrated around blood vessels and their complementary large airways where lesions originate. A gradient influx of these immune cells into AD is evident at P150, before AC expansion and invasion.

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**Fig. 2. Zeb1 and EMT mark small foci of AC generation.** (A) Foci (F) of E-cadherin–negative cells, consistent with EMT, appear at P150 in the central tumor (38). (B) These foci cells are Zeb1hi. (C and D′) Zeb1hi foci cells give rise to Zeb1lo AC cells that migrate away and form adjacent papillary structures (outlined by arrows). (E to G) 4′,6-diamidino-2-phenylindole (DAPI) staining to highlight heterochromatin and Zeb1 expression distinguishes AD, foci (F) and AC cells. (H to H‴) Two foci (F) of AC cells generation at P150, as in (D), were coimmunostained for Zeb1 and Tgfb1. Note that the loss of surrounding Tgfb1 coincides with down-regulation of Zeb1 as AC cells migrate away from foci and form papillary structure (highlighted by arrows). (I) At P190, clusters of invading Zeb1hi cells appear at the IF. Immunostaining for Tgfb1 shows that these clusters are surrounded by Tgfb1. “C” is central tumor and “IF” is invasive front. Scale bars, 100 μm.
surrounding these early invading Zeb1hi, PDL1 + cell clusters (Fig. 3, C). The number of these cells was significantly reduced in areas immediately surrounding invading AC cells. Although a gradient influx of M1-like macrophages from regions of AD persisting at the tumor edge extending from underlying AC cells in the central tumor. Notably, immune cell composition surrounding these Zeb1hi, Tgfb1-rich invading AC cell clusters, was distinct from that seen surrounding adjacent AD and AC cells in the central tumor (fig. S2). CD8+ cells and cells expressing M1-like macrophage markers were largely excluded from the immediate microenvironment surrounding invading clusters, and cells expressing markers of M2-like macrophages were adjacent to the invading clusters. These results suggest a switch to an anti-inflammatory—like environment immediately surrounding invading cell clusters. Below, we link this immune cell composition change to Zeb1-dependent induction of PDL1 and CD47 checkpoints on invading AC cells.

PDL1 and CD47 induction is linked to reprogramming the local immune microenvironment surrounding invading AC cells

By P190, Zeb1hi invading AC cells surrounded by Tgfb1 begin emerging at the lesion edge, and we found that these cells had induced PDL1 (Fig. 3, A and B”). As noted above, CD8+ cells were enriched at the AD edge with penetration into the central lesion (fig. S1), but the number of these cells was significantly reduced in areas immediately surrounding these early invading Zeb1hi, PDL1+ cell clusters (Fig. 3, C and H, and fig. S2). These findings are consistent with a T cell checkpoint consisting of PDL1 on invading AC cells binding to PD1 on the CD8 cells to inhibit their expansion in the microenvironment of invading cell clusters.

CD47 has been referred to as a “marker of self”, and CD47 on red blood cells, circulating hematopoietic stem cells and leukemia cells binding to Sirpa on macrophages, signals a block in macrophage phagocytosis of these cells (46, 47). CD47 was evident at a low, near background level throughout lesions, but like PDL1, it was induced specifically on emerging clusters of Zeb1hi invading cells at the tumor edge (Fig. 3, D to D”). Notably, despite expression of Zeb1 and evidence of EMT, these invading AC cells are easily distinguished from surrounding AD because they were reduced immediately surrounding invading AC cells at the tumor edge (Fig. 3, D to D”). As noted above, CD8+ cells were enriched at the AD edge with penetration into the central lesion (fig. S1), but the number of these cells was significantly reduced in areas immediately surrounding these early invading Zeb1hi, PDL1+ cell clusters (Fig. 3, C and H, and fig. S2). These findings are consistent with a T cell checkpoint consisting of PDL1 on invading AC cells binding to PD1 on the CD8 cells to inhibit their expansion in the microenvironment of invading cell clusters.

Next, we used immunostaining for inducible nitric oxide synthase (iNOS) and CD80 to follow proinflammatory M1-like macrophages. Although a gradient influx of M1-like macrophages from surrounding uninvolved lung into AD was evident with enrichment at the lesion edge, these M1-like cells were excluded from the immediate microenvironment of invading Zeb1hi, CD47hi AC cell clusters (Fig. 3, D, G and H, and figs. S1 to S3). Immunostaining for CD206 and Arg1 was used to follow M2-like macrophages. In contrast to M1-like macrophages, the unininvolved lung, central tumor, and tumor edge were largely devoid of M2-like macrophages at this age, but these cells were closely associated with the emerging invading cell clusters (Fig. 3, F to F” and H, and figs. S1 to S3).

Zeb1 directly induces expression of PDL1 and CD47 mRNAs

We analyzed the effect of Zeb1 knockdown and overexpression on PDL1 and CD47 mRNAs in cell culture. Primary cultures of cells from K-Ras–driven mouse tumors (38, 44) were evaluated by real-time polymerase chain reaction (PCR) (Fig. 3I). Previously, we showed that this Zeb1hi population was CD44hi, Bmi1+, Vim+, E-cadherin+, and Pten+, resembling the expression profile of tumor-initiating cells in foci (38). When this cell population was transferred to the lung, invasive AC developed, and overexpression of Zeb1 in the cells led to metastasis of tumor cells to lymph nodes and other organs (48).

We show that overexpression of Zeb1 causes induction of mRNAs for both PDL1 and CD47. Knockdown of Zeb1 in these cells eliminated PDL1 and CD47 mRNAs, demonstrating that Zeb1 is required for their expression. Promoter regions of CD47 and PDL1 both contain consensus Zeb1 binding E boxes (fig. S4), and chromatin immuno-precipitation (ChIP) assays showed that Zeb1 is bound to both gene promoters in vivo (Fig. 3I). Notably, Zeb1 can act as a transcription repressor of epithelial specification genes, such as Cdh1, via interaction with the corepressor CtBP (49). However, it also acts as a transcriptional coactivator of mesenchymal genes, such as Vim, through its recruitment of TGFb-activated Smads to target genes (50).

Zeb1, PDL1, and CD47 induction continues to mark AC cells as they invade from the tumor edge into surrounding lung

The number of invading AC cell clusters increased by P200, and Zeb1, PDL1, and CD47 continued to mark cells in these invasive clusters (fig. S5). By P220, regions of invading AC cells at the invasive front had extended into surrounding lung parenchyma and alveolar space, and these invading cells retained Zeb1hi, whereas the central tumor remained Zeb1lo (Fig. 4, A and A’). PDL1 continued to be expressed with Zeb1 on the invading cells, and it remained low in the central tumor and in AD cells persisting at the tumor edge (Fig. 4, A’ to B’). CD8+ cells continued to be abundant in unininvolved lung tissue surrounding invasive AC cells and in the central tumor, but they were diminished in localized regions of AC cell invasion (Fig. 4, C to D’, and fig. S2). Lesions in Zeb1 mutant lungs remained AD at this age, which failed to induce PDL1 and PD1+ CD8+ cells continued to be present in the lesions with elevated numbers evident at the lesion edge (Fig. 4, E to G, and fig. S2). This pattern of CD8 cells in AD at P220 in Zeb1 mutant lungs then resembled that seen in early Zeb1 WT lesions at P150 before the appearance of Zeb1hi invading AC cells (figs. S1 and S2).

As with PDL1, CD47 continued to be coinduced with Zeb1 on invading AC cells at P220 (Fig. 4, H to I’, and fig. S2). In addition, cells expressing M2-like macrophage markers continued to be enriched at local sites of invasion, where they were adjacent to Zeb1hi AC cells (Fig. 4, K and K’). However, CD47 expression and these M2-like macrophages remained low in the central tumor as well as regions of AD persisting at the tumor edge (Fig. 4, H to L’, and fig. S2). Coimmunostaining for CD47 on tumor cells and Arg1 on M2-like macrophages showed the close association of the two cell types (Fig. 4, L to L’). M1-like macrophages continued to be abundant in unininvolved lung tissue surrounding tumors, but as with CD8 cells, they were reduced immediately surrounding invading AC cells at this age (Fig. 4, M and N, and fig. S2). In addition, as with CD8 cells, AD persisting in Zeb1 mutant lungs at P220 continued to show M1-like macrophages throughout lesions, with lack of M2-like macrophages anywhere around the lesion edge (Fig. 4, O to Q, and fig. S2).

Results are quantified in Fig. 4R.

As tumors progress, airways become surrounded by tumor cells, and by P220, AC cells had invaded into these airways (Fig. 5A). As with surrounding unininvolved lung parenchyma and alveolar space, inflammatory infiltrate was abundant surrounding and within these airways before AC cell generation (Fig. 1A). Thus, AC cells surrounding and invading airways are also moving into a inflammatory...
environment. As with cells invading into surrounding lung parenchyma and alveolar space, these airway-invading AC cells coinduced Zeb1 and CD47 (Fig. 5, A to C'). Although M2-like macrophages were largely excluded from tumor regions surrounding airway invasion, they nevertheless were enriched within airways at these localized sites of invasion where they were adjacent to Zeb1hi and CD47hi tumor cells (Fig. 5, C'' to F). Again, M1-like macrophages displayed an opposing pattern. They were present in tumor regions surrounding airway invasion but were excluded from airway invasion itself (Fig. 5G). With Zeb1 mutation, persisting AD cells surrounding airways did not induce CD47, M2-like macrophages did not accumulate, and AD cells were not evident within airways (Fig. 5G). We suggest that, as with cell clusters invading into surrounding parenchyma and alveolar space at the tumor edge, Zeb1, via induction of PDL1 and CD47, is reprogramming the local immune microenvironment to an immunosuppressive state at sites of airway invasion. There was little evidence of M2-like macrophages surrounding airways, so we suggest that abundant M1-like cells in this region...
become polarized toward immunosuppressive M2-like cells. Notably, as with AC cells invading into normal lung tissue on the lesion edge, the Zeb1 inducer Tgfβ1 accumulated in and around airways at P190, before invasion of Zeb1hi tumor cells (fig. S6). Tgfβ1 was also evident in adjacent blood vessels at this age, implying that circulating cells are a source of Tgfβ1, along with stromal cells at the tumor edge (51) and that expanding tumor cells induce Zeb1 as they encounter Tgfβ1 at airways.

Together, these results suggest that AD is a proinflammatory environment highlighted by a gradient influx of CD8 cells and M1-like macrophages into the lesion, with cell number higher at the tumor edge than the central tumor. The combination of CD8 cells and M1-like macrophages accounted for most CD45+ cells within lesions, demonstrating that these are the primary immune cell populations moving into the tumors. Because M1-like macrophages secrete cytokines that promote migration and activation of T cells, CD8 cells in surrounding uninvolved lung likely follow M1 macrophages into early lesions where they expand. By contrast, M2-like macrophages secrete factors that inhibit T cell migration and activation, so beyond activation of the PDL1-PD1 axis, the paucity of CD8 cells in

Fig. 4. Zeb1, PDL1, and CD47 coincide with reprogramming of the immune microenvironment around invading AC cells. (A to B”) Zeb1hi and PDL1hi cells at the IF and invading lung parenchyma (PI) at P220. (C) CD8 cells surrounding tumors at P220. (D and D’) CD8 cells at the IF and PI, but not C. (E) PDL1 in a Zeb1 mutant background at P220. (F and G) CD8hi and PD1hi cells in Zeb1 mutant tumors at P220. (H to I) CD47 and Zeb1 on cells at the IF and PI. (J) Arg1hi cells surrounding tumors at P220. (K and K’) Arg1hi cells adjacent to Zeb1hi tumor cells (arrows). (L to R”) Close proximity of CD47hi and Arg1hi cells at the IF at P220. (M) iNos+ cells in normal lung surrounding tumors and airway infiltrate at P220. (N) Few iNos+ cells are evident at the IF at P220. (O) iNos+ cells in tumors in a Zeb1 mutant background. (P to P‴) Most CD68hi macrophages in Zeb1 mutant tumors express iNos. (Q) Few Arg1hi cells are evident in Zeb1 mutant tumors. (R) Effect of Zeb1 on cells in the C, IF, and PI regions of tumors. Regions were identified and counted as in Fig. 3. Error bars are SDs. Scale bars, 100 μm. nd, not detected.

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the microenvironment of invading AC cell might be linked to M2-like cell accumulation at these sites. The localized enrichment of M2-like macrophages in the immediate microenvironment of invading AC cell clusters raised the possibility that CD47 on AC cells binding to adjacent macrophages is driving alternative polarization of macrophages to augment the PDL1-PD1 axis in suppression of CD8 T cell recruitment and expansion at sites of invasion.

**Contact of CD47 on Zeb1 hi AC cells with bone marrow macrophages induces M2-like polarization**

Our results in vivo are consistent with the notion that macrophage contact with Zeb1 hi cancer cells drives their M2-like polarization. To directly test this concept, bone marrow macrophages were co-cultured with primary AC cells from mouse lung tumors, and real-time PCR was used to follow expression of mRNA for the M2-like marker, Arg1 (Fig. 6A). The tumors cells did not express Arg1 mRNA nor did the bone marrow macrophages. However, Arg1 mRNA was induced in cocultures to a level similar to that seen when the bone marrow macrophages alone were treated with M2 polarization inducer interleukin-4 (IL-4) (Fig. 6A). Viral overexpression of Zeb1 in the cancer cells further increased CD47 mRNA (Fig. 3I), and it led to an increase in Arg1 and CD206 mRNAs in coculture (Fig. 6A). To determine whether macrophage proximity to a cancer cell was required for their expression of M2-like markers Arg1 and CD206, cocultures were immunostained and expression was compared in macrophages adjacent and distal to a cancer cell (Fig. 6, B to J). We found cancer cell proximity was required for induction of Arg1 and CD206 on the macrophages, and antibody blocking of CD47 on the cancer cells prevented induction of Arg1 and CD206 on adjacent macrophages, whereas a control antibody did not reduce expression. Together with our in vivo results above, these findings provide evidence Zeb1-dependent induction of CD47 on AC cells is responsible for driving M2-like polarization of adjacent macrophages.

**Fig. 5. Zeb1 and CD47 coincide with reprogramming of the immune microenvironment around AC cells invading into of AWs.** (A) H&E section showing invasion of an AW at P220. (B to B‴) Coinduction of Zeb1 and CD47 at sites of AW invasion. (C to E.Controllers proximity of cells expressing CD47 and Arg1 at sites of AW invasion. (F) Close proximity of Zeb1 hi AC and Arg1+ macrophages at sites of AW invasion. (G) Quantification of cells expressing Zeb1, CD47, iNos, and Arg1 within AWs and surrounding AWs. Immunostaining in Zeb1 mutant lesions and for iNos is not shown. Three representative microscopic views (~200 µm in diameter) were counted for each region within a single tumor. In addition, results from three tumors from different lungs were averaged. As noted above, Zeb1 mutation prevents AD-to-AC transition, and persisting AD do not show evidence of AW invasion or induction of CD47 around AWs. In addition, note Zeb1 is not expressed in AD cells. Error bars are SDs. Scale bars, (A to D‴ and F) 100 µm and (E to E″) 25 µm.
**M2-like macrophages at the invasive tumor front express PDL1**

M2-like macrophages at the invasive tumor front were found to express PDL1. This polarization is induced by cancer cells through CD47 signaling. The study shows that M2-like macrophages express PDL1 at the invasive front, whereas M1-like macrophages in the central tumor are PDL1-negative. This suggests that PDL1+ and Arg1+ cells outnumber PDL1- and Arg1- cells in the region (Fig. 6N).

**DISCUSSION**

Checkpoint immunotherapy is revolutionizing treatment for previously refractive tumors. However, only a fraction of patients respond, and some responders gain resistance. There is intense interest in how resistance to immunotherapy arises, and multiple factors linked to resistance are being identified. One resistance factor is M2-polarized TAMs, which secrete immunosuppressive cytokines that inhibit T cell recruitment and activation, thereby serving as a second barrier to T cell recruitment and expansion in tumors following immunotherapy. A key question under investigation is how TAMs become alternatively polarized in cancer. A second factor predicting resistance to immunotherapy is EMT. EMT is linked to cancer cell invasion, its role in immunotherapy resistance would be focused at the stage of cancer cell invasion and presumably on the invading cells. It is unclear whether these two seemingly disparate resistance factors might be linked.

We followed CD8 cells and macrophages in tumor microenvironments at different stages of K-Ras–driven lung cancer. Initial AD formation elicited a large proinflammatory response throughout the lung containing CD8+ T cells and M1-like macrophages. A gradient influx of these cells into AD was evident (Fig. 6O). Zeb1 mutation prevented transition of AD to AC, and these immune...
microenvironments were maintained as AD persisted and expanded with age in the Zeb1 mutant lungs. In Zeb1 WT lungs, tumor cell invasion initiated with induction of Zeb1 and EMT on clusters of AC cells at the tumor edge. These early invasive clusters coincided with PDL1, and they remodeled their immediate immune microenvironment to exclude CD8 cells (Fig. 6O). Like PDL1, CD47 was also induced with Zeb1 on these invading cell clusters. In addition, as with CD8 cells, M1-like macrophages were excluded from this local microenvironment of the tumor. By contrast, M2-like macrophages were closely apposed to AC cells in these invading clusters. Together, these results show that invasion initiates immunosuppressive reprogramming of the microenvironment surrounding invading clusters (Fig. 6I). In addition, our results linking Zeb1 to induction of CD47 on cancer cells and macrophage polarization support the notion that a CD47-based axis is driving local alternative polarization of macrophages. We cannot rule out that CD47 might be secreted from the cancer cells as a soluble factor to drive adjacent macrophage reprogramming. Such a switch in polarization also induces PDL1 on the macrophages, and the collective induction of PDL1 on both M2 macrophages and tumors cells would target PD1 on surrounding CD8 cells. Furthermore, M2-like macrophages secrete factors that inhibit T cell recruitment and activation, suggesting that they are responsible for the CD8 cell desert surrounding invading tumor cell clusters. Similarly, in lung squamous cell carcinoma, M2 macrophages have been shown to exclude CD8 cells from tumor islets at the invasive edge (53). Illustrating the importance of CD8 cell number at the invasive front of tumors, the degree of increase in CD8 cells at the invasive tumor front following PD1-PD1 blockade is predictive of patient outcome (6). It is of note that we had to rely on histology to guide analysis of the lung tumors due to heterogeneity, and this raises the important issue that similar analysis of heterogeneous human tumors will also require such guided analysis.

Our results highlight invading cancer cells as targets of immune checkpoint therapy, and because Zeb1 induces these checkpoints, its expression in these invading cells might predict patient response to immunotherapy.

**Materials and Methods**

**Study design**

Sample size: Sample numbers are noted in figure legends. P values are presented for all measurements. Rules for stopping data collection: Ages for data collection are noted in figures. Data inclusion/exclusion criteria: No data were excluded. Outliers: Outliers were not excluded. Selection of endpoints: Ages of mice evaluated were based on previous studies showing onset of AD, initiation of AC, and invasion of AC. Replicates: The number of experimental replicates is provided in the figure legends, and P values are provided. Research objectives: The objectives of this study were to evaluate immune cell influx into lesion microenvironments as K-Ras–initiated mouse lung tumors progress from AD to AC; to compare expression of Zeb1, PDL1, and CD47 during tumor progression; to determine linkage between Zeb1/EMT and expression of PDL1 an CD47; and finally to determine whether Zeb1 induction of CD47 was driving M2 polarization of adjacent macrophages. Research subjects or units of investigation: Genetically modified mice were used for the studies, and they are described in Materials and Methods and previous publications.

**Bone marrow–derived macrophage coculture**

Bone marrow was extracted from femurs and tibias of C57BL/6 mice as described (54), and 4 × 10⁴ bone marrow cells were differentiated into bone marrow–derived macrophages (BMDMs) by culturing in one 10 cm² petri dish for 7 days in RPMI supplemented with 10% fluorescence correlation spectroscopy (FCS) and murine recombinant macophagy colony-stimulating factor (M-CSF, 25 ng/ml). Four days after plating, spent medium was replaced with fresh RPMI/FCS/M-CSF for the remaining 3 days. For mRNA expression analyses, 8 × 10⁵ BMDMs were plated in RPMI/10% FCS into each well of a 12-well plate and, 16 hours later, 393P-Zeb1 cells were resuspended in RPMI/10% FCS and plated at 2 × 10⁵ cells per well. Forty-eight hours later, cells were lysed and mRNA isolated and analyzed by quantitative PCR (qPCR). For in vitro coculture staining, 3.6 × 10⁵ BMDMs were plated in RPMI/10% FCS on chamber slides (~45,000 cells per well) for 16 hours followed by plating of 393P-Zeb1 cells resuspended in RPMI/10% FCS at 2 × 10⁵ cells/ml (25,000 cell per well). Cells were cocultured in the absence or presence of neutralizing anti-CD47 monoclonal antibody (mAb, 10 μg/ml) or control mAb (BioXCell) for 48 hours and then immunostained as described.

**RNA analysis**

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized using the Invitrogen RT Kit according to the manufacturer’s protocol (Invitrogen). SYBR Green real-time qPCR was performed using the Mx3000P Real-Time PCR System (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions. PCR primers are shown in table S1. Three independent samples, each in triplicate, were analyzed for each real-time PCR condition, and products were analyzed for size by agarose gel. Patient RNA expression data for human non–small cell lung cancer were obtained from the National Center for Biotechnology Information database (GSE_11969). Data were corrected for background and normalized to median fluorescence and β-actin mRNA levels.

**ChIP assays**

ChIP assays using formaldehyde to cross-link genomic DNA were performed as we have described (37, 55). The chromatin was mechanically sheared to an average length of 200 to 700 base pairs (bps). Rabbit polyclonal antiserum for Zeb1 was used for immunoprecipitation, whereas equal amount of preimmune serum was used as a control [immunoglobulin G (IgG)]. Immunoprecipitation with histone 3 antibody (H3) included in an EpiTect ChIP antibody kit (Qiagen, catalog no. GAH-2206) was used as a positive control. Sequences of primers for target promoters and the expected size of the PCR products are shown in table S1. ChIP-PCR programs were similar to that described below for qPCR, but with additional 1% bovine serum albumin and 1% dimethyl sulfoxide, and the PCR programs usually had a higher annealing temperature (e.g., 60° to 68°C) and longer extension time (e.g., 1 min). Results are representative of three independent experiments.

**Zeb1 shRNA lentivirus knockdown**

We have described lentiviral shRNA knockdown of Zeb1 in detail previously (37, 56–58). To generate a lentivirus expressing Zeb1 shRNA, a primer containing a Zeb1 shRNA sequence (5’-CTGCTTAG-AACAAAAAGAGACATGGAAAATCTGATTGGAATTTG- CAAACAGTGGTCTTGGGATCTGTCCTCATAA-3’) was used with a T3 primer to amplify a 500–base pair fragment
containing the H1 promoter using the pSuper vector as a template. The resulting PCR product was digested with SpeI and Xbal and cloned back into the lentiviral vector digested with Nhel. We first cloned the shRNA sequence into a cytomegalovirus–green fluorescent protein (GFP) lentiviral vector, where its expression was driven by the mouse U6 promoter. Briefly, the shRNA construct was generated by synthesizing an 83-mer oligonucleotide containing (i) a 19-nucleotide sense strand and a 19-nucleotide antisense strand, separated by a 9-nucleotide loop (TTCAAGAGA); (ii) a stretch of five adenines as a template for the Pol III promoter termination signal; (iii) 21 nucleotides complementary to the 3’ end of the Pol III U6 promoter; and (iv) a 5’ end containing a unique Xbal restriction site. The long oligonucleotide was used, together with a sp6 oligonucleotide (5’-ATTAGGTGACACTATAGAAT-3’), to PCR-amplify a fragment containing the entire U6 promoter plus shRNA sequences; the resultant product was digested with Xbal and SpeI ligated into the Nhel of the lentivirus vector, and the insert was sequenced to ensure that no errors occurred during the PCR or cloning steps. Lentiviral particles were produced by a four-plasmid transfection system. Briefly, 293T cells were transfected with the lentiviral vector, and the insert was sequenced to ensure that no errors occurred during the PCR or cloning steps. Lentiviral particles were produced by a four-plasmid transfection system. Briefly, 293T cells were transfected with the lentiviral vector and packaging plasmids, and the supernatants, containing recombinant pseudotemploviral particles, were collected from culture dishes on the second and third days after transfection. Beyond this original shRNA, we used five additional shRNA lentiviruses with different ZEB1 shRNA sequences from Open Biosystems for knockdowns with similar effects. Lentiviruses with a scrambled shRNA sequence (5’-CAACAAGATGAAGAGCACCAATCTCTTGAAT TGGTGCTCTTCATCTTGTTG-3′) was used as a control—this sequence was used to yield K-Ras LA1 , Zeb1lo AC cells. Cells in regions of surrounding AD were counted in three different fields. Again, three mice were analyzed and results were averaged. Foci of AC generation: Three microscopic views (~200 μm in diameter) over three invasive clusters were counted.

Immunostaining

Immunostaining of lung tumors was described in detail previously (37, 38). Briefly, slides were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 10 min, then washed again with PBS and treated with methanol at −20°C for 10 min, and blocked with 4% goat at room temperature for 1 hour. Slides were incubated with primary antibody overnight at 4°C. The next day, slides were washed with PBS followed by incubation with secondary antibody for 1 hour at room temperature. Antibodies for immunostaining are described in table S1. Fluorescent images were captured using an Olympus FV300 confocal microscope (Olympus Confocal America Inc., Center Valley, PA). Images shown are maximum projections of confocal stacks, adjusted for contrast and brightness with Adobe Photoshop Elements v9.0.2 (Adobe Systems Inc., San Jose, CA) and transferred to PowerPoint for figure assembly. As a negative control, no immunostaining was evident in the absence of primary antibodies.

Cell culture

When lung tumors from K-Ras, p53 compound mutants, were placed in culture, a small subset of tumor cells survived and proliferated in culture, and these cells were highly tumorigenic when delivered intracheally back into WT lungs (38, 44). These cells were grown in DMEM with 10% heat-inactivated fetal bovine serum. We found that this population was Zeb1+ , CD44hi , Bmi1+ , E-cadherin− , and Pten− (37). Zeb1 overexpression and shRNA knockdown was described previously (38, 59).

Mutant mice

Housing and handling of all mice was in accordance with procedures approved by the University of Louisville IACUC. K-rasLA1 mice (36) in a C57BL/6 background were obtained from the Jackson laboratory. These mice were crossed with Zeb11/− mice (55) also in a C57BL/6 background to yield K-RasLA1 , Zeb11/−/− and K-RasLA1 , Zeb11/−/+ mice. Lungs were obtained for analysis of tumors at the indicated ages. PCR genotyping was as described previously (37). Tumor pathology was evaluated blindly by an experienced pathologist (M. Cu). On the basis of our previous studies, we did not detect any differences with regard to sex, and thus, male and female mice were chosen at random.

Cell quantification and statistical analysis

Central, invasive edge, and peripheral invasion into parenchyma and alveolar space: Three microscopic views (~200 μm in diameter) were counted in three representative sites in the central tumor at the edge/invasive front and peripheral invasion in a single tumor. This counting was then repeated in tumors from three different mice in Zeb1 WT and mutant backgrounds as indicated in figure legends. These results were averaged, and SDs are presented. Significance was determined by the Student’s t test. Sites of early invasion at P190: Microscopic views (~200 μm in diameter) over three invasive clusters were counted. In addition, views were counted in the central tumor at this age and at the tumor edge 300 μm on either side of the invasive cluster (see diagram in Fig. 3H). These views did not contain invading cell clusters. The counts were then repeated in three different mice at P190 and the results were averaged. Foci of AC generation: Three microscopic views (~200 μm in diameter) over different Zeb1hi foci cells were counted. In these same views, cells were also counted in the adjacent Zeb1lo AC cells. Cells in regions of surrounding AD were counted using three different fields. Again, three mice were analyzed and results were averaged.

Error bars in figures represent SDs. Significance was determined by the Student’s t test. Investigators analyzing immunostaining and tumor analysis were blinded to mouse genotype.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/21/eabd7455/DC1

REFERENCES AND NOTES

1. T. E. Keenan, K. P. Burke, E. M. Van Allen, Genomic correlates of response to immune checkpoint blockade. Nat. Med. 25, 389–402 (2019).

Downloaded from http://advances.sciencemag.org/ on May 28, 2021

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drives immune evasion in genetically reconstituted colon cancer metastasis. Nature **554**, 538–543 (2018).

27. L. C. de Erausco, M. Zuaoua, H. Arasanaz, A. Bocanegra, C. Hernandez, G. Fernandez, M. J. Garcia-Granda, E. Blanco, R. Vera, G. Kochan, D. Escors, Resistance to PD-L1/PD-1 blockade immunotherapy: A tumor-intrinsic or tumor-extrinsic phenomenon? Front. Pharmacol. **11**, 441 (2020).

28. A. Veillette, J. Chen, SIRPα–CD47 immune checkpoint blockade in anticancer therapy. Trends Immunol. **39**, 173–184 (2018).

29. M. J. Kwon, A. Bens, Mouse models for lung cancer. Mol. Oncol. **7**, 165–177 (2013).

30. C. Navas, I. Hernández-Porras, A. J. Schuhmacher, M. Sibilia, C. Guerra, M. Barbacid, EGF receptor signaling is essential for K-ras oncogene-driven pancreatic ductal adenocarcinoma. Cancer Cell **22**, 318–330 (2012).

31. M. S. Song, L. Salmena, P. P. Pandolfi, The functions and regulation of the PTEN tumour suppressor. Nat. Rev. Mol. Cell Biol. **13**, 283–296 (2012).

32. G. Jin, M. J. Kim, H. S. Jeon, J. E. Choi, D. S. Kim, E. B. Lee, S. I. Cha, G. S. Yoon, C. H. Kim, T. H. Jung, J. Y. Park, PTEN mutations and relationship to EGFR, ERBB2, KRAS, and TP53 mutations in non-small cell lung cancers. Lung Cancer **69**, 279–283 (2010).

33. K. Iwanaga, Y. Yang, M. G. Raso, L. Ma, A. E. Hanna, N. Thilagathan, S. Moghaddam, C. M. Evans, H. Li, W. W. Cai, M. Sato, J. D. Minna, H. W. C. J. Creighton, F. J. Demayo, I. I. Wistuba, J. M. Kuri, Pten inactivation accelerates oncogenic K-ras-initiated tumorigenesis in a mouse model of lung cancer. Cancer Res. **68**, 1119–1127 (2008).

34. M. D. Muzumdar, J. K. Dorans, K. M. Chung, R. Robbins, T. Tammela, Y. Gocheva, C. M. Li, T. Jacks, Clonal dynamics following p53 loss of heterozygosity in Kras-driven cancers. Nat. Commun. **7**, 12685 (2016).

35. E. L. Jackson, K. P. Olive, D. A. Tuveson, R. Bronson, D. Crowley, M. Brown, T. Jacks, The differential effects of mutant p53 alleles on advanced murine lung cancer. Cancer Res. **65**, 10280–10288 (2005).

36. L. Johnson, K. Mercer, D. Greenbaum, R. T. Bronson, D. Crowley, D. A. Tuveson, T. Jacks, Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature **410**, 1111–1116 (2000).

37. Y. Liu, X. Lu, L. Huang, W. Wang, G. Jiang, K. C. Dean, B. Clem, S. Teleng, A. B. Jensen, M. Cuatrecasas, J. Chesney, D. S. Darling, A. Postigo, D. C. Dean, Different thresholds of ZEB1 are required for Ras-mediated tumour initiation and metastasis. Nat. Commun. **5**, 5660 (2014).

38. Y. Liu, L. Siles, X. Lu, K. C. Dean, M. Cuatrecasas, A. Postigo, D. C. Dean, Mitotic polarization of transcription factors during asymmetric division establishes fate of forming cancer cells. Nat. Cell Biol. **9**, 2424 (2018).

39. S. Mainardi, N. Mijolli, S. Francoz, C. Vicente-Duenas, I. Sanchez-Garcia, M. Barbacid, Identification of cancer initiating cells in K-Ras driven lung adenocarcinoma. Proc. Natl. Acad. Sci. U.S.A. **111**, 255–260 (2014).

40. K. D. Sutherland, J. Y. Song, M. C. Kwon, N. Proost, J. Zevenhoven, A. Bens, Multiple cells-of-origin of mutant K-ras-induced mouse lung adenocarcinoma. Proc. Natl. Acad. Sci. U.S.A. **111**, 4952–4957 (2014).

41. X. Xu, L. Huang, C. Futtner, B. Schwab, R. R. Rampersad, Y. Lu, T. A. Sporn, B. L. M. Hogan, W. M. Onaitis, The cell of origin and subtype of K-Ras-induced lung tumors are modified by Notch and Sox2. Genes Dev. **28**, 1929–1939 (2014).

42. U. Dehner, J. Schubert, U. C. Burk, O. Schlhalhofer, F. Zhu, A. Sonntag, B. Waldvogel, C. Vannier, D. Darling, A. zur Hausen, V. G. Brumont, J. Morton, O. Sansom, J. Schuler, M. P. Stennaker, C. Herberz, U. Hopt, T. Keck, S. Brabottel, T. Brablet, The EMT-activator ZEB1 promotes tumorigenicity by repressing stromas-inhibiting microRNAs. Nat. Cell Biol. **11**, 1487–1495 (2009).

43. Y. Yang, Y. H. Ahn, Y. Chen, X. Tan, L. Guo, D. L. Gibbons, C. Ungewiss, D. H. Peng, X. Liu, S. H. Lin, N. Thilagathan, I. I. Wistuba, J. Rodriguez-Canales, G. McLendon, C. J. Creighton, J. M. Kuri, ZEB1 sensitizes lung adenocarcinoma to metastasis suppression by PI3K antagonism. J. Clin. Invest. **124**, 2966–2978 (2014).

44. Y. H. Ahn, D. L. Gibbons, D. Chakravarti, C. J. Creighton, Z. H. Rizvi, H. P. Adams, A. Pertschukidis, P. A. Gregory, J. A. Wright, G. J. Goodall, E. R. Flores, J. M. Kuri, ZEB1 drives metastatic prometastatic actin cytoskeletal remodeling by downregulating miR-34a expression. J. Clin. Invest. **122**, 3170–3183 (2012).

45. C. Kröger, A. Afeyan, J. Mraz, E. N. Eaton, F. Reinhardt, Y. L. Khodor, P. Thiru, B. Bierie, X. Ye, C. B. Burge, R. A. Weinberg, Acquisition of a hybrid E/M state is essential for tumorigenesis of basal breast cancer cells. Proc. Natl. Acad. Sci. U.S.A. **116**, 7353–7362 (2019).

46. P. A. Oldenberg, A. Zeleznikay, Y. F. Fang, C. F. Lagenaur, H. D. Gresham, F. P. Lindberg, Role of CD47 as a marker of self on red blood cells. Science **288**, 2051–2054 (2000).

47. Z. Bian, L. Shi, Y. L. Guo, Z. Lv, C. Tang, S. Niu, A. Tremblay, M. Venkataramani, C. Culpepper, L. Li, Z. Zhou, A. Mansour, Y. Zhang, A. Gewirtz, K. Kidder, K. Chen, Y. Liu, Cd47-Sirpα interaction and IL-10 constrain inflammation-induced macrophage phagocytosis of healthy self-cells. Proc. Natl. Acad. Sci. U.S.A. **113**, E5434–E5443 (2016).

48. A. Etzrodt, K. Tsakitsinis, M. Maniecki, W. Damsky, M. Dellini, E. Baudoin, M. Moulin, M. Rosenberg, J. H. Graversen, N. Vaphianis-Annez, S. K. Moestrup, T. Lawrence, Specific...
targeting of CD163(+) TAMs mobilizes inflammatory monocytes and promotes T cell-mediated tumor regression. J. Exp. Med. 216, 2394–2411 (2019).

49. A. A. Postigo, D. C. Dean, ZEB represses transcription through interaction with the corepressor CBP. Proc. Natl. Acad. Sci. U.S.A. 96, 6683–6688 (1999).

50. A. A. Postigo, J. L. Depp, J. J. Taylor, K. L. Kroll, Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. EMBO J. 22, 2453–2462 (2003).

51. M. Cortés, L. Sánchez-Moral, O. de Barrios, M. J. Fernández-Aceñero, M. C. Martínez-Campanario, A. Esteve-Codina, D. S. Darling, B. Györffy, T. Lawrence, D. C. Dean, A. Postigo, Tumor-associated macrophages (TAMs) depend on ZEB1 for their cancer-promoting roles. EMBO J. 36, 3336–3355 (2017).

52. D. Murooka, N. Seo, T. Hayashi, Y. Tahara, K. Fujii, I. Tawara, Y. Miyahara, K. Okamori, H. Yagita, S. Imoto, R. Yamaguchi, M. Komura, S. Miyano, M. Goto, S. I. Sawada, A. Asai, H. Ieda, K. Akiyoshi, N. Harada, H. Shiku, Antigen delivery targeted to tumor-associated macrophages overcomes tumor immune resistance. J. Clin. Invest. 129, 1278–1294 (2019).

53. E. Peranzoni, J. Lemoine, L. Vineux, V. Feuillet, S. Barrin, C. Kantari-Mimoun, N. Bercovici, M. Guérin, J. Biton, H. Ouakrim, F. Reginer, A. Lupo, M. Alfano, D. Darnotte, E. Donnadieu, Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti-PD-1 treatment. Proc. Natl. Acad. Sci. U.S.A. 115, E4041–E4050 (2018).

54. M. B. de Souza Rizzo, M. B. de Carvalho, E. J. Kim, B. E. Rendon, J. T. Noe, A. D. Wise, R. A. Mitchell, Oral squamous carcinoma cells promote macrophage polarization in an MIF-dependent manner. QJM 111, 769–778 (2018).

55. Y. Liu, S. El-Naggar, D. S. Darling, Y. Higashi, D. C. Dean, Zeb1 links epithelial-mesenchymal transition and cellular senescence. Development 135, 579–588 (2008).

56. Y. Liu, E. Sánchez-Tilló, X. Lu, B. Clem, S. Telang, A. B. Jenson, M. Cuatreras, J. Chesney, A. Postigo, D. C. Dean, Rb1 family mutation is sufficient for sarcoma initiation. Nat. Commun. 4, 2650 (2013).

57. Y. Liu, E. Sánchez-Tilló, X. Lu, H. Huang, B. Clem, S. Telang, A. B. Jenson, M. Cuatreras, J. Chesney, A. Postigo, D. C. Dean, The ZEB1 transcription factor acts in a negative feedback loop with miR200 downstream of Ras and Rb1 to regulate Bmi1 expression. J. Biol. Chem. 289, 4116–4125 (2014).

58. Y. Liu, X. Peng, J. Tan, D. S. Darling, H. J. Kaplan, D. C. Dean, Zeb1 mutant mice as a model of posterior corneal dystrophy. Invest. Ophthalmol. Vis. Sci. 49, 1843–1849 (2008).

59. H. Lu, K. R. Clauser, W. L. Tam, J. Fröse, X. Ye, E. N. Eaton, F. Reinhardt, V. S. Donnenberg, R. Bhargava, S. A. Carr, R. A. Weinberg, A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. Nat. Cell Biol. 16, 1105–1117 (2014).

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Zeb1 induces immune checkpoints to form an immunosuppressive envelope around invading cancer cells

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