Review of the pathologic assessment of immune response in lung cancer

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Abstract: Lung cancer is the most common cause of cancer death worldwide. While targeted therapies have offered promise for a small subset of patients with driver mutations, usually found among light/never-smokers, the majority arise among heavy smokers are usually not benefited. Immune checkpoint inhibition has instead offered greater promise in this larger cohort of patients. This observation has led to greater attention to the immune components of the tumor microenvironment (TME) and a model of tumor immunoediting has emerged in which cancer evolves through phases of elimination, equilibrium, and escape. The chief effector cell in this process is the cytotoxic T-cell (CTL) which can be measured quantitatively using immunohistochemistry for CD8. While the density of this population within primary tumors and their metastasis is prognostic in advanced stage disease of both small cell lung carcinoma (SCLC) and non-small cell lung cancer (NSCLC), the prognostic significance is more variable in early stage disease and in particular among adenocarcinoma (LUAD) which are heterogenous in their morphology, biology, and risk factor associations. The anti-tumor role of CTL is dependent on a host of immune cell interactions which can be measured by assessing tumor-infiltrating lymphocytes (TIL) on routine H&E staining as well as specific dendritic cell populations associated with the formation of tertiary lymphoid structures (TLS) using immunohistochemistry for LAMP or regulatory T-cells (Treg) using FoxP3. Finally, tumor associated macrophages (TAM) and neutrophils (TAN) may polarize to promote (M1 or N1) or inhibit (M2 or N2) CTL effectiveness and can be measured using a variety of immunohistochemical approaches. Herein we review the immunopathologic features of TME in lung cancer and their prognostic associations.

Keywords: Tumor associated macrophage (TAM); tumor associated neutrophil (TAN); tertiary lymphoid structure (TLS); tumor-infiltrating lymphocyte (TIL); non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer is the most common cause of cancer death worldwide and cigarette smoking is the most significant risk factor (1). In recent years immune checkpoint inhibitors have offered promise for a subset of lung cancers with high tumor mutational burden associated with neoantigen formation that commonly occur in the setting of long-standing carcinogenic exposure. As our understanding of the interaction between cancer and the immune systems

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has grown, the classical Hallmarks of Cancer (2) have been updated to include the category of "immune evasion" (3). Immune evasion is now understood as the process of cancer immunoediting whereby immune cells interact with neoplastic cells in phases of elimination, equilibrium, and finally escape as neoplastic cells evolve into fully malignant tumors (4,5). Cytotoxic T-cells (CTLs) are the chief effector cell in this process but their effectiveness is regulated by a variety cytokines and cell-to-cell interactions between immune cells residing in regional lymph nodes as well as immune cells recruited to the tumor site. While the inflammatory features may be ascertained by pathologic analysis of resected tumor, the effects are also observed in systemic inflammatory markers such as CRP (6). The pathologic features of the tumor localized immune response in lung cancer will be the subject of this review.

Methods

PubMed search 2000–2020, using keywords of lung cancer and TIL, TAM, TAN, Treg, B-cell or TLS was performed. The search is further narrowed down by selecting review articles involving human lung cancer, and peer reviewed papers with discussion of clinical outcome.

Cancer immunoediting

The elimination phase of lung cancer is difficult to visualize given that such specimens do not usually come to pathologic examination. Since the advent of CT-lung screening (CTLS), there has become an increasing awareness that high-risk smokers often have one to several pulmonary nodules below the size criteria for therapeutic intervention (7). While some of these nodules progress to cancer many others remain stable or else disappear on follow up exams. While disappearing lesions are assumed to be inflammatory, some of these may be in fact represent inflammatory reactions successfully eliminating early cancers.

The equilibrium phase represents the point where neoplastic growth and immune related inhibition reach equilibrium such that a lung nodule becomes stable. Some of these nodules represent slow-growing indolent cancers while others are high-grade tumors which are undergoing selection for clones that can breach the wall of CTL immune response. While unusual in its clinical presentation, rare cases of small cell lung carcinoma (SCLC) are detected by CT-scan at early stage and treated by surgery prior to receiving chemotherapy. Investigators from Finland have published a series of 56 operable SCLC (66% stage I/II) and showed that high density of CD8 positive CTL were associated with small tumor size, early stage, and favorable prognosis (8). Examples of two cases of SCLC are depicted in *Figure 1* in which H&E and immunohistochemistry for CD8 show striking differences in the degree of CTL infiltration between a case without metastasis (*Figure 1A*,*B*) to another cases that showed widespread metastasis within 3-months of initial detection (*Figure 1C*,*D*). While rare, these early stage SCLC with high CTL infiltration serve to illustrate the equilibrium phase of an aggressive cancer.

The escape phase represents the point where a tumor is able to grow and metastasize in spite of the tumor immune response. Previous investigators have systematically evaluated this phenomenon among resected non-small cell lung cancer (NSCLC) that subsequently metastasized. Comparing the degree of CD8 positive CTL, they showed that brain metastasis from NSCLC patients have lower CD8 T-cells than their paired previously resected primary lung tumors (9). Interestingly, decreased CD8 T-cells in brain metastasis correlated with shorter overall survival (9) implying that an ongoing dynamic between metastatic subclones and the host immune system may persist beyond the initial escape phase from the primary tumor site.

Tumor infiltrating lymphocytes (TIL)

The immune portion of the tumor microenvironment (TME) is made up of a host of multiple immune cell types; including neutrophils, macrophages, dendritic cells, natural killer (NK) cells, T cells and B cells. TIL is the predominant portion of immune TME which include both lymphocytes and plasma cells. TIL can be divided into three compartments: (I) lymphocytes within cancer cell nests (epithelial lymphocytes), (II) lymphocytes in the central cancer stroma (stromal lymphocytes), (III) lymphocytes present along the invasive margins (peritumoral lymphocytes) (10). In most studies there has been an improved survival associated with density of TIL although the criteria used to assess TIL has varied.

A series of published cohorts using criteria originally proposed by Pittsburgh have compared TIL– (none to only scattered lymphocytes within the stroma) to TIL+ (moderate to intense stromal lymphocytic infiltrate with variable intraepithelial lymphocytes) (11-13). TIL+ cases made up 42–47% of NSCLC using their criteria and improved outcomes were seen in stage IA (11), larger (>5 cm) organ confined tumors (12), as well as locally advanced stage IIIA

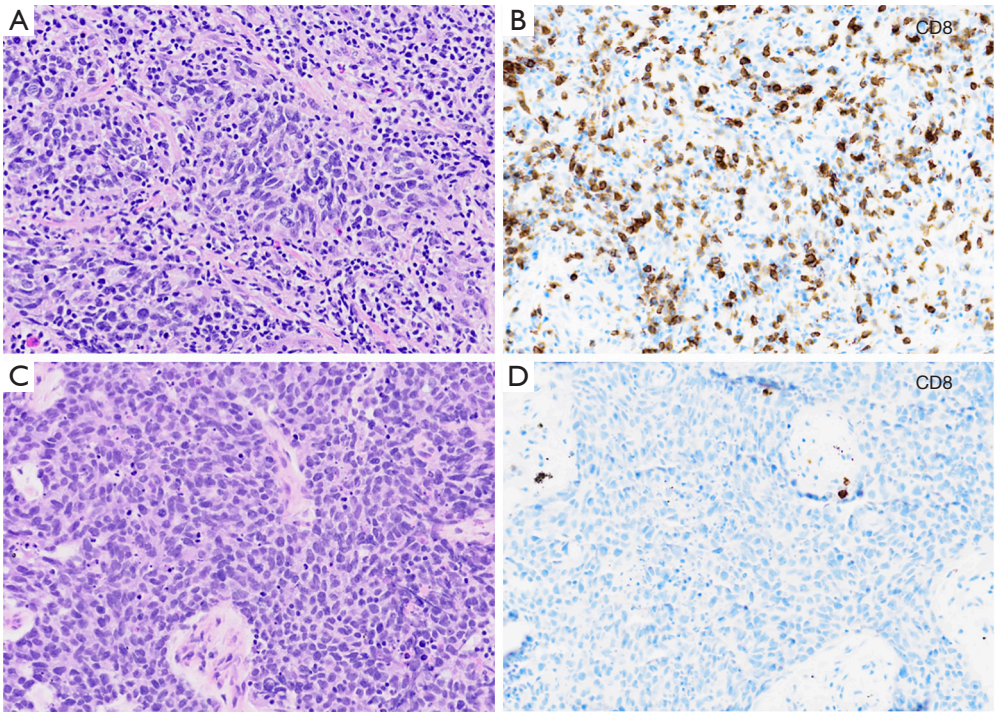

Figure 1 Cytotoxic T-cell Infiltrate in SCLC. (A) H&E and (B) CD8 staining of a case of early stage SCLC without metastasis showing a brisk CTL infiltrate. (C) H&E and (D) CD8 staining of a case of early stage SCLC with widespread metastasis within 3-months of diagnosis and only sparse CTL infiltrate. Original magnification ×200. SCLC, small cell lung carcinoma; CTL, cytotoxic T-cell.

(N2) tumors (13). In the later study, subset analysis showed that TILs were prognostic in lung squamous cell carcinoma (LUSC) but not lung adenocarcinoma (LUAD).

Subsequent studies have applied the standardized methods of TIL assessment proposed for solid tumors by the International Immuno-Oncology Biomarkers Working Group (14,15). In this assessment the stromal and intra-tumoral compartments are scored separately and reported as a continuous percentage of lymphoplasmacytic cells occupying the stromal or tumoral area respectively. Lepidic foci in which no desmoplastic reaction is present, intra-alveolar tumor clusters (spread through air spaces, STAS) and alveolar macrophages are not scored (14). Examples of varying degrees of TIL are shown in *Figure 2*. Stromal TIL (sTIL) as thus defined have been assessed in three NSCLC cohorts where intense sTIL (>50%) was observed in 9–22% of cases (16-18). In a series of 537 stage I–III NSCLC, increasing sTIL (0–5%, 6–25%, 26–50%, >50%) was associated with improved disease-specific survival (DSS), disease-free survival (DFS), and overall survival (OS) at each threshold (16). On subset analysis, the prognostic significance of these sTIL thresholds were maintained for advanced stage NSCLC with a non-significant trend for stage I disease; whereas sTIL was not associated with LUAD outcomes (16). In a larger series of 1,546 stage I–III NSCLC, intense sTIL was similarly associated with improved DFS, DSS, and OS with a similar effect observed in both LUAD and LUSC however comparison of early *vs.* advanced stage in these subtypes was not reported (17). Finally, in a series of 146 stage I–III LUAD intense sTIL was associated with better progression-free survival (PFS) as well as OS which remained significant on subset analysis for advanced stage LUAD but with only a non-significant trend for stage I LUAD (18).

It is noteworthy that the impact of sTIL appears less significant for LUAD than LUSC and is only observed when greater densities of sTIL are used. In the two studies specifically examining advanced stage disease (13,18) including LUAD, the proportion of TIL+ cases by Pittsburgh criteria (42%) (13) was notably higher than observed by the intense sTIL criteria (22%) (18). Both populations were Asian [Chinese (13) and Korean (18)], with high rates of light/never-smokers [48% (13) and 58% (18)] in which EGFR mutations are known to be more frequent (19)

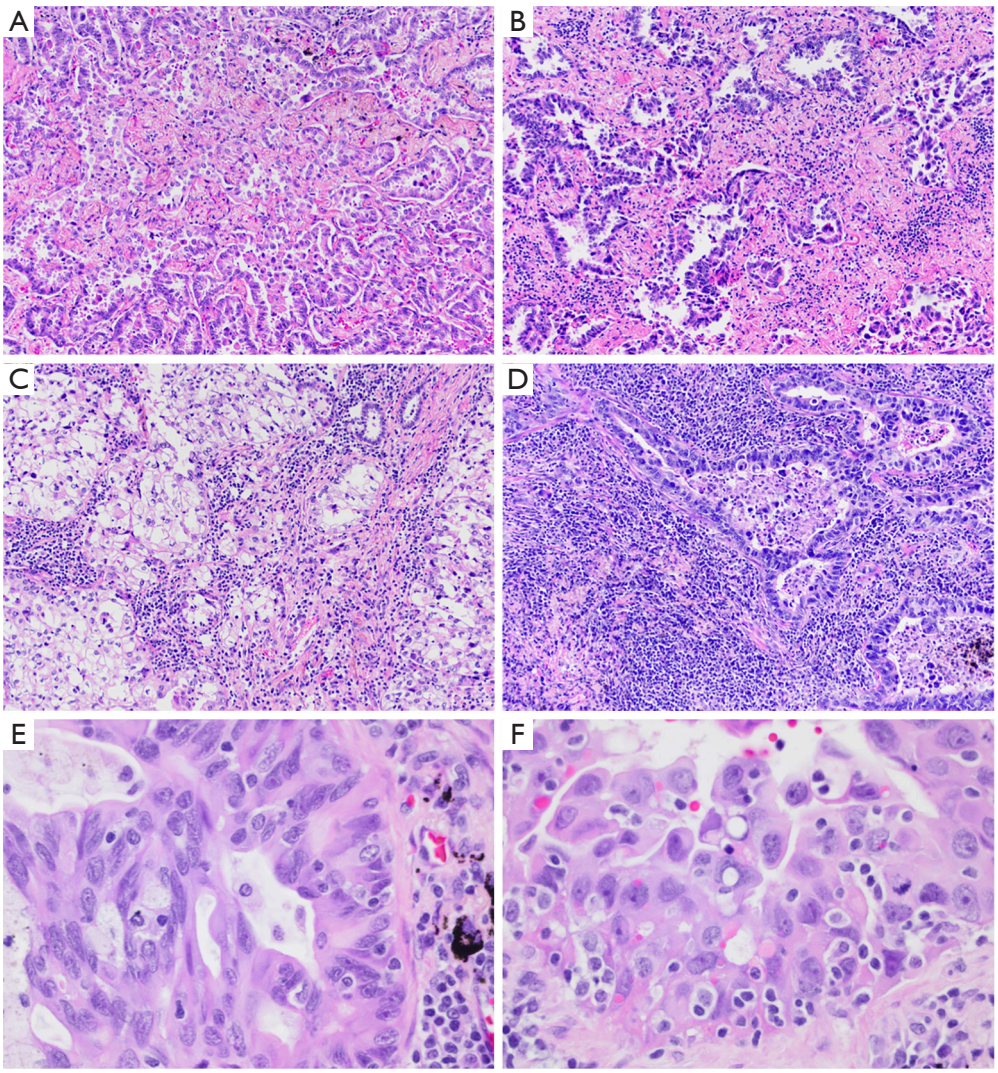

Figure 2 Spectrum of tumor infiltrating lymphocytes (TIL) in NSCLC. H&E stained sections showing (A) sparse <5%, (B) mild 6–25%, (C) moderate 26–50%, and (D) intense >50% stromal TIL as well as (E) mild and (F) marked intra-tumor TIL. Original magnification ×200 (A,B,C,D), ×400 (E), ×500 (F).

and specifically assessed at 54% (18) in the latter study. In this context others have shown that improved survival for sTIL above the median value (17%) was more pronounced for KRAS mutant rather than EGFR mutant LUAD (20). In aggregate, these data suggest that cutoff values for TIL may be dependent on the underlying biology of the tumor which likely effects the proportion of expressed neoantigens. Moreover, the same concept may explain why the degree of prognostic benefit of sTIL is greater in advanced stage disease than in early stage disease since a subset of stage I LUAD are slow growing and behave in an indolent fashion (21).

CTL

As mentioned previously, CTL represent the primary effector cell immune response against tumor. Using digital imaging of immunohistochemical stained slides for CD3 and CD8, an Immunoscore assessment for colon cancer has been widely validated as prognostic across tumor stage with implications for altering the TNM classification to include Immunoscore results (TNM-I) (22,23).

To this aim, the density of stromal CD4 and CD8 positive T-cells were observed to be positive prognostic factors on multivariant analysis in LUSC but not LUAD in

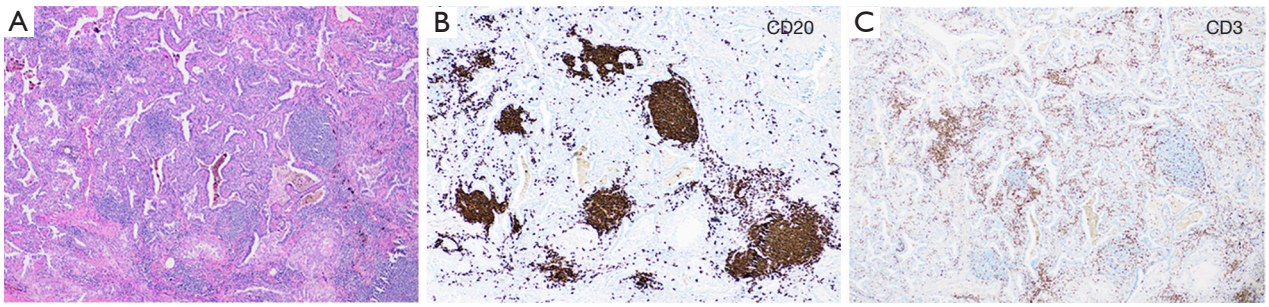

Figure 3 Tertiary lymphoid structures in non-small cell lung cancer (NSCLC). H&E stained sections showing (A) lung adenocarcinoma (LUAD) with nodular aggregates of lymphocytes. Most of the nodules stain with (B) CD20 while smaller nodules are seen staining with (C) CD3. Original magnification ×40.

a series of 335 resected NSCLC stage I–IIIA (10). Using a combination of CD45RO to identify memory T-cells in the tumor (tCD45RO) and CD8 in the stroma (sCD8), these same authors showed that combined high-tCD45RO/high-sCD8 was a stage independent positive prognostic factor in LUSC using a series of 536 NSCLC stage I–IIIA (24). Finally they expanded their work to include 797 stage I–IIIA NSCLC and showed the high-sCD8 T-cell density served as a single positive prognostic marker across stages with LUAD now also reaching statistical significance in this larger cohort but with a lower magnitude than LUSC (25). From these data, they proposed a TNM-Immunoscore for resected NSCLC (26). Stage specific assessment of LUAD was not performed in these studies; however, in a larger cohort of 956 stage I LUAD assessed by a separate group, neither tumor nor stromal densities of CD4 or CD8 were prognostic (27). It should be noted that each of these studies have been performed using tissue microarrays (TMA) rather than on whole tumor sections as would be required to bring the Immunoscore into clinical practice as has been suggested for colon cancer.

While densities of CTL appear prognostic, their activation and clonal expansion requires interaction with a host of immune cells in the TME. The most well studied components of the TME in NSCLC include tertiary lymphoid structures (TLS), regulatory T-cells (Tregs), tumor associated macrophages (TAM) and tumor associated neutrophils (TAN).

TLS

TLS are organized lymphoid aggregates representing de novo lymphoid neogenesis at sites of chronic inflammation such as infection, autoimmune processes, allograft rejection and solid cancers (28-33). TLS are present in many cancer types (34,35) and are essential for the ongoing generation of effector memory T helper cells, CTL, effector memory CTL, memory B cells and antibody-producing plasma cells (36-39). TLS recapitulate the structure of the lymph node and are composed of B-cell follicles with germinal centers and adjacent nodular T-cell aggregates. Examples of TLS in NSCLC is shown in *Figure 3*. The nodular organization of both B-cell follicles/germinal centers and T-cell nodules is architecturally the result of follicular dendritic cells and LAMP-positive mature dendritic cells (DC-LAMP) respectively (40).

The significance of TLS for NSCLC was first described among a series of 74 early stage NSCLC (46 LUAD and 28 SCC) (41). Histologically these were described as tumor-induced bronchus-associated lymphoid structures (Ti-BALT) and were quantified by immunohistochemically measuring DC-LAMP+. High DC-LAMP+ cases correlated with superior DFS, DSS, and OS as well as the amount of Th1 T-cells staining with T-bet (T-box transcription factor 21) and CTL staining with granzyme B. The authors went on to show in a larger cohort of 376 NSCLC in which immunohistochemistry, flow cytometry of disaggregated tumor tissue, and gene expression profiling that tumors rich in DC-LAMP also exhibited high infiltration by effector memory T-cells (CD45RO+CD8+), greater T-cell activation, a T-helper 1 (Th1) phenotype, and cytotoxic orientation (38). These findings further support the notion that CTL need a host of immune cell interactions within the tumor to maintain antitumor activity.

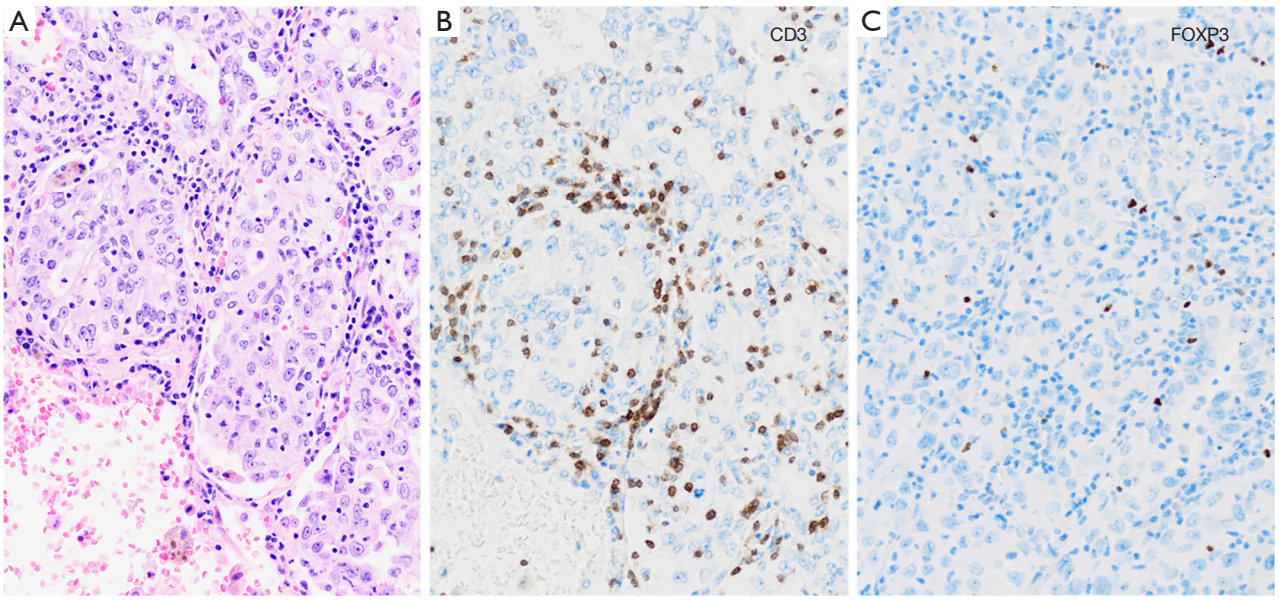

Figure 4 Regulatory T-cells in non-small cell lung cancer (NSCLC). H&E stained sections showing (A) lung adenocarcinoma (LUAD) with mild increase in stromal and tumoral TIL staining with (B) CD3 and a subset staining with nuclear (C) FoxP3. Original magnification ×200.

Treg

Alternatively, Treg function to diminish the hosts immune response against self or foreign antigens whereby they serve an important protective role against the development of autoimmune disease but alternatively can function to quench the host immune response against cancer (42,43). Treg are typically recognized in tissue by immunohistochemistry for forkhead box P3 (FoxP3). *Figure 4* shows an LUAD with proportions of CD3 positive T-cells and FoxP3 positive Treg. The negative prognostic impact of Tregs was shown in a series of 956 stage I LUAD in which a high relative proportion of stromal FoxP3 to CD3 T-cells negatively correlated with outcome (27). Using this index, authors showed a high FoxP3/CD3 index score was associated with high tumor grade, vascular invasion and lymphatic invasion but not tumor size or driver mutation status. Specifically, the index remained prognostic in the subset of LUAD ≤2 cm (27) which may suggest that Treg exert their pro-tumor effects early in the course of immunoediting.

TAM

TAM function as immune regulators in the TME by the production of cytokines, growth factors, and proteases, with both pro- and anti-tumor activity (44). TAM polarize into one of two major populations: proinflammatory M1 (or classically activated) and anti-inflammatory M2 (or alternatively activated) (45,46). M1 macrophages secrete proinflammatory cytokines like IL-6, IL-12, IL-23, express MHC class II and stimulate T-cell–mediated antitumor immunity. The M2 phenotype is induced by Th-2 derived cytokines like IL4, IL10, IL13, transforming growth factor-beta, or prostaglandin E2 (47). M2 macrophages promote tissue repair via supporting immune tolerance, tissue debridement, and remodeling. M2 macrophages may also therefore function to promote tumor growth. Common immunohistochemical markers include the pan-macrophage marker CD68; M1 TAM stain with HLA-DR, iNOS, and pSTAT1; while M2 TAM stain with CD206, CD204, and CD163 (48). Representative images depicting varying degrees of TAM infiltration by routine H&E and sections stained with CD163 are shown in *Figure 5*.

Stromal M2 macrophage density as measured by immunohistochemistry for CD204 was a negative prognostic marker in Japanese cohorts of 170 LUAD stage I–IIIA (49) and 255 LUSC stage I–IIIA (50). Interestingly in a Scandinavian cohort of 553 NSCLC stage I–III using multiplex assays to assess M1 (HLA-DR/CD68), M2 (CD163/CD68 or CD204/CD68), the density of both M1 and M2 stromal and intratumor cells were all associated with improved survival (51). A meta-analysis including

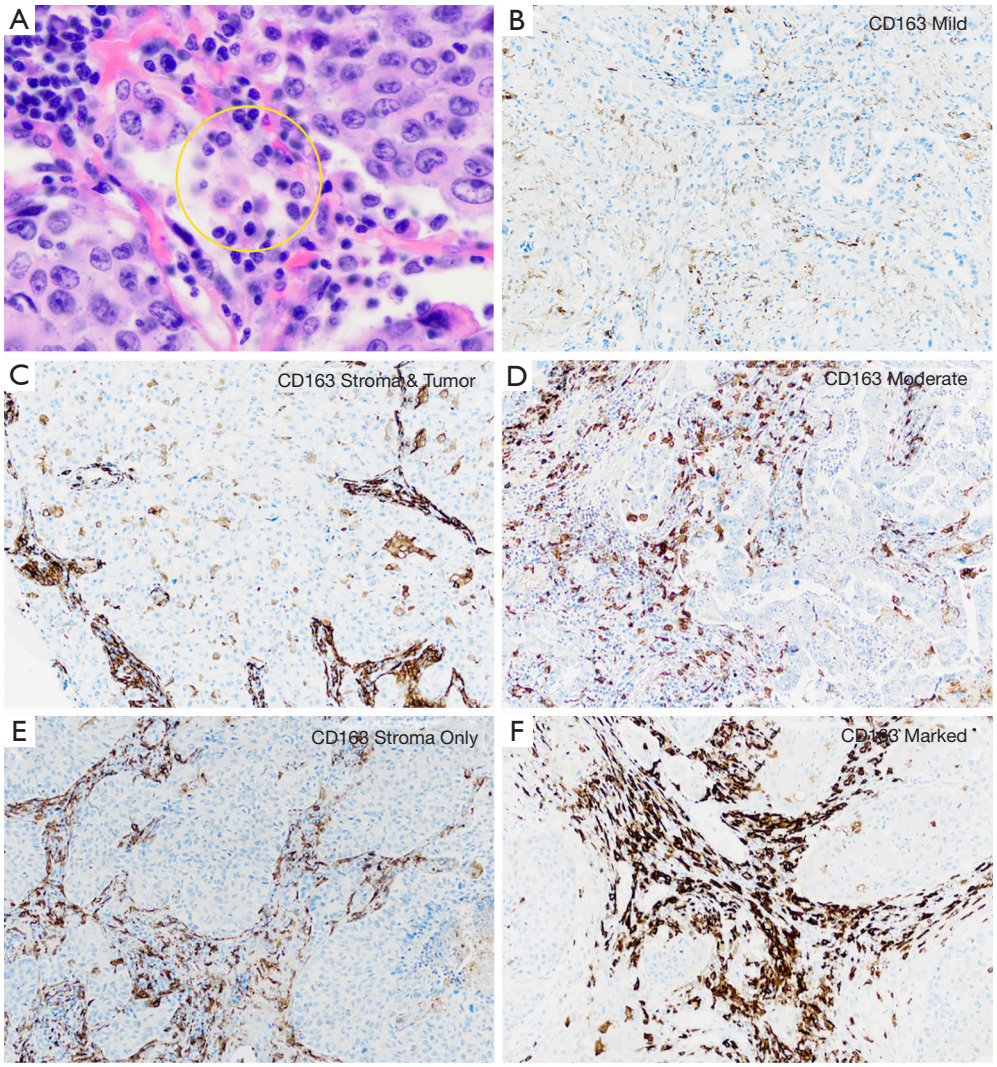

Figure 5 Tumor associated macrophages in non-small cell lung cancer (NSCLC). H&E stained sections showing (A) inconspicuous histiocytes in LUAD. Immunohistochemistry for CD163 shows staining in (C) stroma and tumor and (E) stroma only. Density of CD163 stromal staining ranging from (B) mild, (D) moderate, to (F) marked. Original magnification ×400 (A), ×100 (B,C,D,E,F).

2,572 NSCLC from 20 different studies concluded that a high density of intratumor M1 TAM was associated with improved OS while high density of stromal M2 TAM was associated with a poor OS (52). The differences observed in these studies may reflect different population types but also likely reflect the specificity of the method of immunohistochemical detection of M1 *vs.* M2 TAM.

TAN

Neutrophils make up a variable portion of the immune infiltrate in a wide variety of cancer types (53). Neutrophils, similar to macrophages, have been subcategorized into anti-tumor (N1-neutrophils) and pro-tumor (N2-neutrophils). Extensive work in early stage NSCLC using enzymatically digested tumor samples has been performed to characterize both the phenotype by flow cytometry and effect on T-cells using ex vivo functional assays (54). Two subtypes of TAN have emerged from this work: activated canonical TANs ($CD11b^{+}$, $CD66b^{+}$, $CD15^{hi}$, $HLA\text{-}DR^{-}$, $CD14^{-}$) and non-canonical antigen presenting cell (APC)-like hybrid TANs ($CD11b^{+}$, $CD66b^{+}$, $CD15^{hi}$, $HLA\text{-}DR^{+}$, $CD14^{+}$) (55). Both subtypes exhibit an anti-tumor effect by stimulating T-cell immune response however hybrid TAN appear to

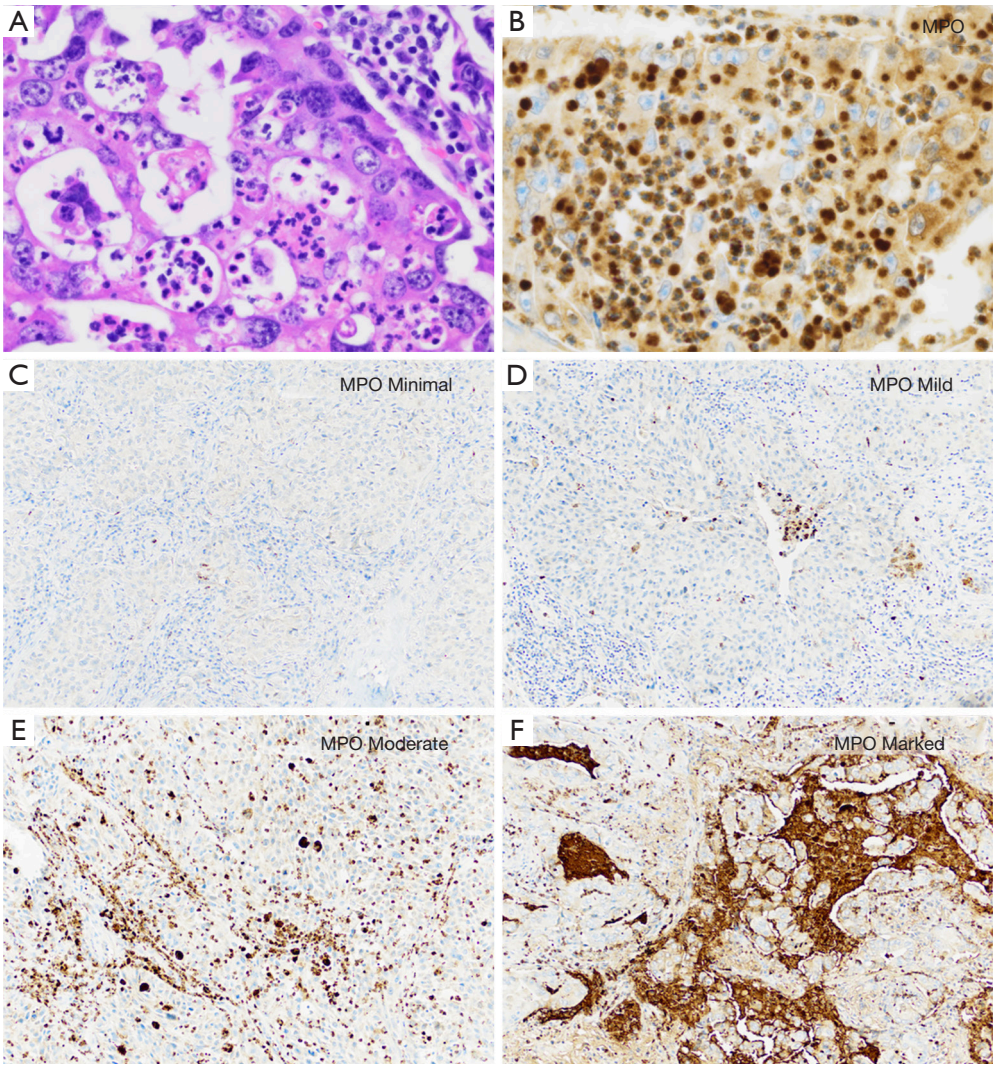

Figure 6 Tumor associated neutrophils in non-small cell lung cancer (NSCLC). H&E stained sections showing (A) dense neutrophilic infiltrate with LUSC which stain with (B) myeloperoxidase (MPO) by immunohistochemistry. MPO staining ranging from (C) minimal, (D) mild, (E) moderate, to (F) marked are shown. Original magnification ×400 (A,B), ×100 (C,D,E,F).

contribute the most to amplification of anti-tumor effector CTL responses in early stage tumors (54,56). The later finding is intriguing given that hybrid TAN frequency is inversely proportional to tumor size, suggesting that immune escape occurs as hybrid TAN density decreases. Myeloid-derived suppressor cells represent another phenotypic subset of neutrophils with T-cell inhibition and pro-tumor function but these are not easily recognized by routine pathologic methods (53,57).

Pathologically, neutrophils are readily identifiable by routine H&E staining however quantification by immunohistochemistry is the preferred method. The two most commonly used antibodies are myeloperoxidase (MPO) and CD66b. MPO is a peroxidase enzyme located within the granules of neutrophils and released into tissue upon degranulation while CD66b is a cell adhesion molecule found on the surface of human granulocytes. Representative images depicting varying degrees of neutrophil infiltration by routine H&E and sections stained with MPO are shown in *Figure 6*.

Three separate studies have examined the quantity of neutrophils enumerated by immunohistochemical staining of TMAs from large databases of surgically resected NSCLC. In a French study of 632 resected NSCLC stage

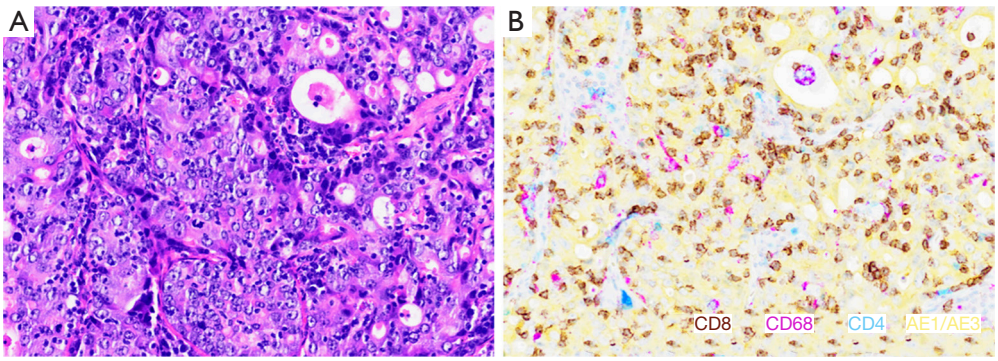

Figure 7 Multiplex chromogenic immunohistochemistry in non-small cell lung cancer (NSCLC). H&E stained sections showing (A) cribriform LUAD with marked tumoral and mild stromal increase in TIL. Multiplex immunohistochemistry (B) shows tumor cells in yellow (AE1/AE3), CTL in brown (CD8), T helper cells in teal (CD4), and macrophages in purple (CD68). Original magnification ×200.

I–III (58), CD66b neutrophil density was associated with higher frequency of recurrence but not with histologic type, stage, grade, or smoking status. Used in combination with the quantity of CD8 positive CTL, the CD66b-positive neutrophil-to-CD8-positive lymphocyte ratio (iNTR) using a cutoff of >1 (30% of the cohort) showed a higher frequency of recurrence and worse overall survival, remaining significant on multivariant analysis second only to stage as a predictor of worse outcome. While CD66b staining alone cannot distinguish a N1 from an N2 TAN, the diminished CD8 count was interpreted an indirect measure of anti-tumor effect and thus the authors suggested this ratio may indicate an increase in N2 TAN's in these cases. Two other studies examining the density of CD66b staining apart from the ratio with CD8 have shown variable results. In a series of 536 resected NSCLC stage I–IV, poor outcome was only observed in LUAD but not LUSC (59) whereas another group studied CD66b staining in 335 resected NSCLC stage I–IIIA and found no prognostic significance (60). Taken together, the aggregate findings suggest that the poor prognostic effect of increased neutrophils in NSCLC is related to their inhibitory effects on CTL, suggesting a "N2-like" interaction albeit without a definitive immunohistochemical marker to prove this supposition.

Conclusions and future directions

The complexity of the tumor immune response and the dynamic process of cancer immunoediting has added yet another layer of complexity in our understanding of NSCLC. No doubt advanced techniques to include multiplex chromogenic or fluorescent immunohistochemistry or even ion-based immunohistochemistry using imaging mass cytometry (61) will add tremendous insight to the field as the number of antigens detected per cell increases while maintaining the context of their spatial organization. This in conjunction with single cell sequencing approaches will no doubt increase our knowledge further of the individual cells making up the TME (62). An example of multiplex chromogenic immunohistochemistry is shown in *Figure* 7 which demonstrates the potential for assessment of not just quantitative but also spatial data of the immune contexture. Perhaps one day such assays will become standard of care for assessing both prognostic and predictive data to guide therapy in patients with NSCLC.

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