The Role of Inflammation in the Pathogenesis of Non-small Cell Lung Cancer

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Abstract: The link between chronic immune activation and tumorigenesis is well established. Compelling evidence has accumulated that histologic assessment of infiltration patterns of different host immune response components in non-small cell lung cancer specimens helps identify different prognostic patient subgroups. This review provides an overview of recent insights gained in the understanding of the role played by chronic inflammation in lung carcinogenesis. The usefulness of quantification of different populations of lymphocytes, natural killer cells, macrophages, and mast cells within the tumor microenvironment in non-small cell lung cancer is also discussed. In particular, the importance of assessment of inflammatory cell microlocalization within both the tumor islet and surrounding stromal components is emphasized.

Key Words: Inflammation, Non-small cell lung cancer, Immunohistochemistry, Lymphocyte, Macrophage.

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There is now a general consensus that chronic inflammation plays a critical role in tumorigenesis.[1] In response to injury, there is activation of a complex integrated system of mechanisms designed to eliminate the stimulus, repair damaged tissue, and promote wound healing through measured and organized cellular proliferation. Under normal circumstances, this host physiological response subsides once repair is completed. However, when this usually tightly regulated self-limiting mechanism becomes disturbed, a state of chronic inflammation may ensue.

Virchow[2] first proposed the link between inflammation and neoplasia when he observed that cancer seemed to develop at sites of previous chronic inflammation. The initial suggestion that tumor-infiltrating lymphocytes (TIL) may represent an expression of host antitumour activity was supported by evidence linking TIL with improved prognosis in solid cancers.[3,4] Forty years later, there is now a wealth of accumulated data to support the hypothesis that organs affected by chronic inflammation provide the perfect milieu in which an abnormal clone or clones of cells are capable of evading detection by the host immune system and progress to invasive carcinoma.

This review summarizes recent insights gained in the understanding of the role played by chronic inflammation in lung carcinogenesis. The usefulness of various methods of quantification of different inflammatory cell populations within the tumor microenvironment in non-small cell lung cancer (NSCLC) using immunohistochemistry is discussed. In particular, the importance of assessment of inflammatory cell microlocalization within both the tumor islet and surrounding stromal components is emphasized.

CHRONIC IMMUNE ACTIVATION AND CARCINOGENESIS

The adaptive immune response is known to comprise two essential and complementary components: cell-mediated immunity (CMI) and humoral immunity (HI). The $CD4^+$ T-helper (T_H) lymphocyte is an essential element of both systems and is responsible for orchestrating two different but overlapping cytokine patterns that influence other effector cells and in turn shape the pattern of the inflammatory response.[5]

The molecular mechanisms that underlie the evolution of matured naive (T_H0) $CD4^+$ cells into distinct populations that are characterized by either a CMI or HI phenotype are still not completely understood. However, the nature of the cytokine milieu in which antigen presentation occurs influences the differentiation of T_H0 cells. Early production of interleukin (IL)-12 by mature dendritic cells stimulates upregulation of CMI by effector/memory cells and is characterized by the production of the T_H1 cytokines IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α.[6] These cytokines exert antiangiogenic and proapoptotic effects. By contrast, HI is characterized by the production of proangiogenic, antiapoptotic T_H2 cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13.[7] Immune deviation toward a dominant T_H1 response facilitates cancer cell killing and tumor rejection.[8] Conversely, a domi-

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nant T_H2 response may be regarded as favorable for tumor progression.[9]

Under prevailing physiological conditions, a delicate balance exists between CMI and HI responses. In response to an inflammatory stimulus (e.g., exposure to a viral pathogen), there is activation of both systems to clear the injurious stimulus. Once the offending pathogen is successfully removed, and in order for effective wound healing to occur, there is a relative reduction in CMI and upregulation of HI.[10] Thereafter, the normal balance between CMI and HI is reestablished. However, in circumstances where the host is unable to effectively remove the inciting stimulus, the inflammatory cellular components persist and a chronic inflammatory response may develop. Such a pattern is characteristic of a number of human infectious diseases such as Epstein-Barr virus, human papillomavirus, hepatitis B and C, human immunodeficiency virus/acquired immunodeficiency syndrome, *Helicobacter pylori*, and schistosomiasis.[11] A failure of host infection-clearing mechanisms is typical of each of these chronic infections.

Several lines of evidence indicate that many cancers develop in the setting of such chronic immune activation. In these circumstances, there is not only an overall upregulation of immune responses but a shift in the immune response to one that is characterized by a wound healing, proangiogenic, antiapoptotic cytokine pattern. As the malignant process develops, cancer cells evolve to subvert the CMI response. Taken together, these findings highlight the critical role played by the immune system in determining outcome with respect to tumorigenesis.[10,11]

THE ROLE OF CHRONIC INFLAMMATION IN LUNG CANCER PATHOGENESIS

Cigarette Smoking

Cigarette smoking is established as the leading risk factor for lung cancer, and the overwhelming majority of patients diagnosed with the disease are current or former smokers. There is increasing evidence that tobacco smoke exposure promotes widespread inflammatory and mutagenic effects in the lungs that promote a pro-cancer immune response. Indeed, the initial pathologic hallmark of smoking is a widespread inflammatory infiltrate throughout the lung parenchyma.[12] Repeated injury to lung epithelial cells caused by inhalation of noxious particulate matter from cigarette smoke drives ongoing recruitment of host inflammatory cells to lung airways and alveolar tissue. The lungs of smokers are characterized by increased numbers and enhanced activation of macrophages, dendritic cells, activated lymphocytes, and granulocytes, thereby producing an environment conducive to the proliferation of malignantly transformed cells.

Environmental Tobacco Smoke

Environmental tobacco smoke (ETS), also referred to as "passive" or "involuntary" smoking, has been extensively investigated as a potential cause of lung cancer. The National Research Council in the United States estimates that 2 to 3% of all lung cancer (about 3000 cases per year) may be attributable to ETS. Much of epidemiologic research on ETS has examined the relative risk increase among nonsmoking women according to the level of smoking by husbands. Overall, there is an increased risk of at least 20% among nonsmoking wives of smoking husbands when compared with nonsmoking wives of nonsmoking husbands.[14] Prolonged exposure to ETS in the workplace also confers an increased risk and forms the basis for smoking ban legislation in an increasing number of countries.

Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a disorder characterized by an abnormal local and systemic inflammatory response and is strongly associated with lung cancer.[15,16] Within the lung, cigarette smoke incites a potent inflammatory reaction in the airways and alveoli of susceptible smokers, a process etiologically important in driving both proteolytic alveolar destruction and airway remodeling, the pathologic hallmarks of COPD.[17] The inflammatory responses that characterize COPD drive a repetitive cycle of injury and repair throughout the lungs. These persistent biologic effects also increase the risk of transformation of normal bronchial epithelium to a malignant phenotype.

There are data confirming that reduction in lung function, the physiologic hallmark of COPD, is associated with increased markers of systemic inflammation. In particular, levels of forced expiratory volume in one second (FEV_1) show an inverse correlation with levels of C-reactive protein, a sensitive marker of inflammation.[18] Numerous epidemiological studies have evaluated the relationship between lung cancer and abnormalities of pulmonary function, with moderate to severe airflow obstruction now recognized as an independent predictor of incident lung cancer.[19] This risk increases with worsening severity of lung function impairment as measured by FEV_1 and appears particularly strong in women.[20]

Nonsmoking-Related Factors

Exposure to tobacco smoke is indisputably the most important risk factor for the development of both COPD and lung cancer in susceptible hosts. However, approximately 10% of lung cancers occur in lifelong nonsmokers, indicating that other factors must be etiologically relevant in lung carcinogenesis.[21] In this regard, a number of other disorders of the respiratory system in which inflammation is pathogenic, both infectious and noninfectious, have been linked to the development of lung cancer.

Pulmonary Infections

Much of the evidence for the increased risk of lung cancer in the setting of prior infection due to *Mycobacterium tuberculosis* has been derived from large epidemiological studies. A large population-based case-control study conducted in China among individuals with evidence of previous tuberculosis disease showed a positive correlation with both adenocarcinoma and squamous cell cancer subtypes, which was independent of smoking status and

socioeconomic group.[22] Zheng et al.[23] reported that a prior diagnosis of tuberculosis increased the risk of lung cancer by approximately 50%, with the effect most apparent among those with recent infection.

A causal association between lung cancer and *Chlamydia pneumonia* has been the subject of investigation[24] using serologic testing as evidence of prior infection. The precise mechanisms to explain this epidemiological link remain poorly understood. Chronic infection with *C. pneumonia* has been shown to result in impaired apoptosis of infected cells by induction of the key immunosuppressive cytokine IL-10 resulting in reduced antitumor CMI responses.[25] Another postulated mechanism by which *C. pneumonia* may increase risk of malignancy is through upregulation of IL-8 and promotion of angiogenesis and cellular proliferation.[26]

Occupational Dust Exposure

Accumulated epidemiological data have suggested that fibrotic lung diseases may also predispose to lung tumorigenesis. This link was initially suggested by the finding of coexisting interstitial lung disease and lung carcinoma in autopsy studies.[27] The observed increased incidence of lung cancer in follow-up studies of workers exposed to asbestos and silica seems to support this hypothesis.[28,29]

The link between asbestos and lung cancer was first made nearly 70 years ago, an association that was strengthened by epidemiologic data from the 1960s showing that textile workers in Britain exposed to high levels of asbestos dust had a 10-fold increased incidence.[30] The risk conferred by asbestos exposure is increased in a multiplicative manner in smokers because of a synergistic effect of several carcinogens found in cigarette smoke. Results from a European collaborative study confirmed the association between occupational crystalline silica dust exposure and lung cancer, with a twofold increased risk observed among individuals with greatest exposure.[31] Other proven or suspected occupational risk factors include nickel, chromium, beryllium, arsenic, and silica.

Idiopathic Pulmonary Fibrosis

A study examining a cohort of patients with idiopathic pulmonary fibrosis found that the incidence of lung cancer was increased sevenfold compared with controls.[32] This increased risk was independent of cigarette smoking history. It has been suggested that overexpression and subsequent mutation of the p53 gene that occur with inflammation/fibrosis-associated oxidative DNA damage and repair may contribute to the emergence of a pro-tumor environment in patients with idiopathic pulmonary fibrosis.[33]

Experimental Models

Animal experiments have provided additional important evidence of the potential contribution of immune activation to lung carcinogenesis in the context of exposure to inorganic dusts. Using a mouse model, Saffiotti and coworkers showed that intratracheal instillation of chemical particles evokes a potent inflammatory response that eventually leads to the development of NSCLC.[34] Insufflation of particulate silica, a potent lung irritant, causes a brisk granulomatous reaction in the murine bronchial tree. Recruitment of inflammatory cells to affected airways predictably leads to hyperplasia of affected bronchiolar epithelium and type 2 pneumocytes. Subsequently, the mouse lung characteristically undergoes progressive changes through hyperplasia to adenoma formation and, ultimately, carcinoma. Whether or not a similar mechanism accounts for lung cancer development in humans remains to be clarified.

Cyclooxygenase

In recent years, attention has been drawn to the pathogenic role played by the prostaglandins and their upstream regulators of production, the cyclooxygenases (COXs), in the progression of malignant disease.[35] COX consists of two distinct isoforms, COX-1 and COX-2; COX-1 is constitutively expressed and regulates a number of physiological functions, and production of the early response gene COX-2 is induced in response to inflammatory stimuli through the action of various cytokines and growth factors. However, tumor promoters also upregulate COX-2 synthesis, and many of the fundamental components of carcinogenesis and cancer development are known to be mediated via its actions. These include impairment of CMI, apoptosis resistance, cellular proliferation, enhanced angiogenesis, increased invasion, and metastasis.[36]

Increased levels of COX has been demonstrated in the initiation stage of a variety of cancers.[37] Epidemiological evidence indicates that long-term users of nonsteroidal anti-inflammatory drugs (agents that block the proinflammatory effects of COX) have an approximate 50% reduction in risk of colon cancer and may confer reduced risk of lung, esophagus, and gastric cancer.[38,39] Several investigators have confirmed that both NSCLC and associated precursor lesions (adenomatous hyperplasia and carcinoma in situ) are associated with COX-2 overexpression.[40–42] Quantification of COX-2 expression may add useful independent prognostic information in resected NSCLC patients.[43]

EGFR and EML4-ALK Mutations

There is now compelling evidence of the importance of both activating mutations within the EGFR[44] and different variants of the EML4-ALK fusion gene[45] in the pathogenesis of lung cancer among never or former light cigarette smokers. However, the potential etiological relevance of host immune dysregulation with respect to lung cancer among exclusively nonsmokers has not thus far been specifically investigated. In addition, the nature of the immune response in patients with these genetic mutations in the context of lung cancer has yet to be established. Given the heterogeneity of established risk factors already discussed for lung cancer among nonsmokers, it is probable that lung carcinogenesis in this population is multifactorial. Furthermore, in light of the clear importance of immune dysregulation for different forms of human cancer that do not have as clear a link with smoking as does

NSCLC (both as an initiating factor and as a determinant of outcome for different stages of disease), it is likely that aberrant immune responses play an important role in the pathogenesis of disease even in those without significant tobacco exposure. Additional clinical investigation to help establish possible links between genetic mutations that are more frequently observed in those with no or minimal cigarette smoke exposure and immune dysregulation is warranted.

RELATIONSHIP OF INFLAMMATORY CELL INFILTRATE PATTERNS AND PROGNOSIS IN NSCLC

From the earliest stages of cancer development, both a systemic and locoregional inflammatory response is induced in the host. The nature of this response is a critical determinant of whether tumor growth is facilitated or inhibited. Experimental evidence indicates that there is a proliferation of host immune effector populations in response to nascent tumors.[6] It was previously believed that this phenomenon represented a frustrated host antitumor defense mechanism. However, there is now evidence that the developing tumor is capable of adapting this host immune response to create a microenvironment conducive to its own survival and progression.[46]

There is conflicting evidence with regard to inflammatory response patterns in lung cancer and their relationship to survival, with evidence to support both beneficial and negative effects. As with other solid tumors, accumulating data indicate that the nature of the both the innate and adaptive immune responses to neoplastic challenge in the lung is of critical importance. Early investigation suggested that an enhanced lymphoreticular response conferred a survival advantage in lung cancer.[47,48] However, variations in the numbers and clinical characteristics of patients analyzed in subsequent studies, together with considerable heterogeneity in the methods used to categorize and examine the inflammatory cell infiltrate, have meant that a less than clear-cut relationship with prognosis has emerged (Table 1). The specific pattern of this response seems crucial in determining tumor progression, and recent evidence suggests that precise localization of immune cells within different parts of the tumor influences phenotype.[49]

T-Lymphocytes

Abundant intratumoral infiltration by different T-lymphocyte populations is a frequent observation in different forms of human cancers, and this histologic finding has been linked to improved patient survival in numerous studies.[50–53] By contrast, other reports have suggested that increased lymphocytic invasion in cancers predicts worse outcome.[54–56] However, it is clear that a failure of host immune surveillance mechanisms, in which lymphocytes play a pivotal role, is a key step in the early stages of tumor development.

Although prominent T-lymphocyte infiltration is a characteristic finding in many forms of malignancy, functional aberrancy of these cells is often apparent. Several processes may account for this anergy. Alterations of the T-cell receptor (TCR) prevent effective recognition of the major histocompatibility complex (MHC)-antigen complex when T-lymphocytes encounter malignant cells. In addition, decreased expression of signal transduction proteins, blunted proliferative capabilities, and a change in cytokine expression profile have also been observed.[57] As distinct from normally functioning circulating lymphocytes, TILs may respond inappropriately or not at all to conventional activating stimuli.[58,59] Together, these effects result in impairment of effective cancer cell killing mechanisms.

Tumor cell surface antigens and soluble products are recognized and processed by host antigen-presenting cells and result in the activation of specific $CD4^+$ helper T-lymphocyte populations. The nature of the cytokine profile elaborated by tumor cells influences whether the T-cell population that is expanded and recruited to the site of the tumor is a dominant T_H1 or T_H2 pattern. For example, abundant expression of the immunosuppressive cytokine transforming growth factor (TGF)-β promotes maturation of T_H2 $CD4^+$ cells[10] and also directly inhibits cytotoxic $CD8^+$ T-lymphocyte–mediated cancer killing mechanisms.[60] Whether the predominant prevailing immune pattern is the result of tumor effects driven by surrounding stromal constituents or develops as a consequence of interplay between both elements remains uncertain.[46]

There is increasing evidence that the immune system recognizes and mounts a specific, albeit relatively ineffective, antitumor response by recognizing cell surface-specific antigens in NSCLC.[1] The adaptive immune response, comprising T-cells, B-cells and antibodies, has been the focus of extensive research in lung cancer. Flow cytometric analyses have demonstrated that the lymphocyte population within NSCLC is characterized by a predominantly antitumor T_H1 cytokine phenotype.[61] Furthermore, increased infiltration by $CD3^+$, $CD8^+$, and B-type TILs shows a positive correlation with the extent of tumor cell apoptosis using Terminal dUTP Nick End Labeling (TUNEL) techniques to quantify fragmented DNA segments. This relationship seems to be strongest with higher grade tumors and is maintained even in patients with advanced disease.[62]

Data from initial investigation of the prognostic implication of TILs assessed overall counts irrespective of location rather than classifying various infiltrating cell types into different compartments with the tumor mass.[63] Support for the notion that immune cell "microlocalization" within lung tumors may be of greater relevance than absolute numbers per se was provided by a study by Johnson et al.[64] These investigators found that grouping patients defined by different levels of semiquantitatively assessed chronic inflammatory cell infiltrate did not provide useful prognostic information. However, the identification of high numbers of $CD3^+$ T-lymphocytes within cancer islets as opposed to supporting peritumoral stroma predicted a more favorable prognosis. Another study found that determination of microlocalization infiltrative patterns of $CD8^+$ lymphocytes did not seem to provide additional prognostic information, although numbers evaluated in this

TABLE 1. Studies Evaluating the Relationship Between Immune Cell Infiltrate and Prognosis in Non-small Cell Lung Cancer

Author	Study Size	Immune Cell Subtype Studied	TNM Stage	Histological Subtypes	Key Findings
Lee et al.[48]	30	T lymphocytes, plasma cells, neutrophils, macrophages	III	Sq, Ad, LC	Increased stromal lymphocyte count associated with improved survival
Tormanen-Napankangas et al.[62]	84	$CD3^+$ and $CD8^+$ T lymphocytes, B lymphocytes, macrophages	Not defined	Sq, Ad, LC	Increased intratumoral infiltration by $CD3^+$ and $CD8^+$ T lymphocytes and B lymphocytes associated with tumor cell apoptosis but not prognosis
Johnson et al.[64]	95	$CD3^+$ and $CD8^+$ T lymphocytes, B lymphocytes, NK cells, macrophages, Langerhans cells	I–III	Nonspecified NSCLC (97%), SCLC (3%)	Improved prognosis in subgroup with higher intratumoral infiltration of $CD3^+$ T lymphocytes and $S100^+$ Langerhans cells
Trojan et al.[65]	31	$CD8^+$ T lymphocytes	I–III	Sq, Ad, LC	No relationship between intratumoral lymphocyte infiltration and prognosis
Kawai et al.[66]	199	$CD8^+$ T lymphocytes, macrophages, mast cells	IV	Sq, Ad, undifferentiated NSCLC	Improved median survival times in patients with high intratumoral macrophages and $CD8^+$ T lymphocytes treated with adjuvant chemotherapy
Al-Shibli et al.[67]	335	$CD4^+$ and $CD8^+$ T lymphocytes, $CD20^+$ B lymphocytes	I–IIIA	Sq, Ad, LC	High intrastromal $CD4^+$ and $CD8^+$ lymphocyte numbers an independent prognostic factor
Hiraoka et al.[68]	109	$CD4^+$ and $CD8^+$ T lymphocytes	I–IIIA	Sq, Ad, LC, AS, carcinosarcoma[a]	High conjoint $CD4^+$ and $CD8^+$ T lymphocyte stromal infiltration a favorable prognostic factor
Wakabayashi et al.[69]	178	$CD4^+$ and $CD8^+$ T lymphocytes	I–IIIA	Sq, Ad	Higher $CD8^+$ T lymphocyte intratumoral counts associated with shorter 5-yr survival. Increased intrastromal $CD4^+$ T lymphocyte counts a favorable prognostic marker
Petersen et al.[83]	64	$CD3^+$ and $Foxp3^+$ T lymphocytes	I	Ad, Sq, "other"	Higher risk of disease recurrence with high intratumoral regulatory:total T-lymphocyte ratio
Kerr et al.[89]	95	$CD3^+$ and $CD8^+$ T lymphocytes, $CD79^+$ B lymphocytes, NK cells, macrophages, Langerhans cells	I–III[b]	Sq, Ad, AS, LC, SCLC, Car	Increased $CD3^+$ T lymphocytes, macrophages (in tumor islets only) and CD4:CD8 ratio in tumors showing histological appearances akin to regressing malignant melanoma
Takanami et al.[90]	150	NK cells	I–IIIA	Ad	NK cell infiltration (predominantly found in stromal regions) a prognostic factor in univariate analysis only
Villegas et al.[91]	50	NK cells	I–IIIA	Sq	Low numbers of intratumoral NK cells associated with increased risk of death
Welsh et al.[49]	175	Macrophages, mast cells	I–IV	Ad, Sq, "other"	High tumor islet/stromal macrophage and tumor islet/stromal mast ratios independent favorable prognostic indicators
Ohri et al.[101]	40	Macrophages		Sq, Ad, LC, "other"	Tumor islets macrophages in patients with increased 5-yr survival predominantly show a cytotoxic M1 phenotype
Kim et al.[102]	144	Macrophages	I–IV	Sq, Ad, AS, LC	High tumor islet macrophage count independent predictor of improved 5-yr survival
Takanami et al.[103]	113	Macrophages	I–IV	Ad	Greater macrophage infiltration associated with increased microvessel density and worse prognosis; macrophages predominantly identified in stroma
Zeni et al.[104]	50	Macrophages	I–IV	Sq, Ad	Increased IL-10 expression by tumor islet macrophages associated with shorter survival
Imada et al.[122]	85	Mast cells	I	Sq, Ad	Stromal mast cells correlate with angiogenesis assessed by microvessel counts and poor outcome
Tomita et al.[125]	90	Mast cells	I–IV	Ad	Increased overall mast cell infiltration associated with improved 5-yr survival rates postsurgery

[a] $N = 1$.

[b] Four patients had undefined tumor, node, metastasis (TNM) stage.

Ad, adenocarcinoma; AS, adenosquamous; Car, carcinoid; CD, cluster of differentiation; IL, interleukin; LC, large cell carcinoma; M, macrophage; NK, natural killer; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; Sq, squamous.

study were small and immune populations were assessed in a semiquantitative fashion.[65]

A principal antitumour host immunomechanism is direct cytotoxicity of $CD8^+$ T-lymphocytes via the recognition of tumor-associated antigens presented on MHC class I molecules. The degree of $CD8^+$ T-lymphocyte infiltration within cancer nests alone has variously emerged as a positive,[66,67] neutral,[64,68] or negative[69] prognostic marker in previous studies. However, different methods have been used to quantify the lymphoid infiltrates in NSCLC specimens. While some studies have used randomly selected regions for analyzing cell counts, others have focused on those regions that have the highest counts of lymphocytes within regions of tumor epithelium and stroma. This introduces a potential regional analysis bias that might account for some of the discrepancy of published results. Furthermore, tumor specimens from some of these studies were collected before the advent of newer, more sophisticated staging technology such as computed tomography and positron emission tomography imaging modalities. Thus, while patients from previous studies may have been regarded as having early-stage disease that was amenable to surgical resection, undetected metastatic disease may have accounted for some of the differences in outcome and acted as a potential confounder.

Aggregation of different cell types into tumor islets may play an important role in the activation of cytotoxic pathways. Concomitant infiltration by $CD8^+$ and $CD4^+$ TILs seems an indicator of favorable prognosis, suggesting that cellular cooperation between different lymphocyte subsets may be necessary for effective tumor cell killing.[68] In this regard, $CD4^+$ T-cells seem to augment tumor cell antigenicity and susceptibility to lysis by $CD8^+$ TILs via local release of TNF-α and IFN-γ.[70] Similarly, synergy between TILs and tumor associated macrophages (TAMs) may be required for optimum antitumor activity, because a combined high tumor/islet $CD8^+$ count ratio and high tumor/islet macrophage count ratio confer a prognostic advantage.[66]

Several factors have been implicated in the relative lymphocyte anergy that results in impaired T-cell mediated cytotoxicity in lung cancer patients with high numbers of TILs and poor outcomes. Although there is frequently extensive infiltration of $CD8^+$ TILs within tumor nests, it seems that it is mainly the peritumorally located $CD8^+$ cells that are immunologically active as determined by measurement of IFN-γ expression.[65] By comparison, intratumoral effector TILs are inadequately activated and display blunted cytotoxic activity, in turn facilitating cancer progression.

There is increasing evidence to support the hypothesis that NSCLC cells themselves induce a local and systemic HI environment with a predominant T_H2 cytokine pattern.[10] A host of human tumors, including NSCLC, are characterized by overexpression of COX-2 which controls production of different prostaglandin isoforms. The enzyme PGE2 stimulates production of $CD4^+$ T_H2 cells but is a potent inhibitor of T_H1 $CD4^+$ T-cells. Experiments using both cell lines and resected tissue specimens have confirmed that lung cancer cells fail to express the T_H1 cytokines IL-2 and IFN-γ, whereas elaboration of IL-10 is significantly enhanced in response to stimulation with IL-4 and TNF-α.[71] Furthermore, increased serum concentrations of IL-10 predict reduced survival in advanced NSCLC.[72]

The local elaboration of various immunosuppressive cytokines, including IL-10, either from tumor cells themselves or from bordering stromal constituents, may hamper effective cytotoxic mechanisms.[73] Alterations in the TCR, loss of signal transducing proteins, and/or attenuated costimulatory signals from antigen-presenting cells have all been suggested as contributory factors to account for this anergy.[74] Infiltrating TILs are known to demonstrate significantly reduced expression of MHC class II molecules such as HLA-DR 39,[75] whereas cancer cells display reduced expression of class I MHC molecules.[76] Effective cytotoxicity may also be further inhibited by overproduction of various immunosuppressive factors such as TGF-β by lung cancer cells despite a predominant CMI response.[77]

Further contributing to a milieu of immune tolerance, accumulation of $CD4^+CD25^+$ regulatory T cells (Tregs) into the tumor bed of NSCLC is a frequently observed histopathological phenomenon. This population of lymphocytes function to maintain peripheral tolerance via suppression of self-antigen–reactive T-cells. Human Tregs coexpress the CD25 antigen and are identified by the presence of the intracellular marker forkhead box P3 (Foxp3), a transcriptional repressor required for maturation and immunosuppressive functionality.[78] It has been suggested that intratumoral accumulation of Tregs may result in downregulation of T-cells reactive against tumor antigens, thereby creating a more favorable microenvironment for clonal tumor cell expansion. This hypothesis is supported by experimental work by Willimsky et al.,[79] who used an experimental model of spontaneous tumor development to show that Tregs suppress specific cytotoxic antitumor elements.

As is the case in other cancers, increased levels of Tregs in NSCLC correlate with tumor invasiveness and metastatic potential. These cells express constitutively high levels of cell surface CTLA-4, suggesting ongoing activation of neighboring cells.[80] Levels of intratumoral TGF-β are contributed by infiltrating Tregs, although directed inhibitory effects by this population are maintained even in the absence of this cytokine. Effective T-cell activation seems to be specifically suppressed by Tregs through inhibition of IL-2, which results in a selective inhibition of local host immune responses.[81]

The expression of the immunosuppressive mediator Foxp3 is, at least in part, regulated by tumor-derived COX-2, which is constitutively overexpressed in lung cancer. Furthermore, COX-2 inhibition in vivo leads to impairment of Treg function and reductions in the numbers of Foxp3-expressing cells, promoting decreases in tumor size through restoration of effectual host antitumour responses.[82]

To date, there have been relatively few studies that have examined the clinical impact of intratumoral infiltration by T-lymphocytes characterized by a regulatory phenotype in the setting of lung cancer. Woo et al.[74] found increased amounts of tumor-associated $CD4^+CD25^+$ cells in NSCLC

patients with early-stage disease compared with normal controls. An immunosuppressive phenotype was further highlighted by the finding of spontaneous TGF-β secretion among TILs. Among patients with stage I NSCLC in this study, increased intratumoral accumulation of Foxp3$^+$ Tregs relative to total TIL infiltration was associated with increased risk of disease recurrence.[83] However, assessment of Treg counts in isolation did not yield prognostic information. These data suggest that anticancer strategies that specifically target intratumoral Tregs, either through a reduction in numbers or via abrogation of immunosuppressive activity, may prove worthy of future investigation.

Natural Killer Cells

Accumulated experimental data indicate that natural killer (NK) cells display spontaneous cytotoxic activities in the presence of cancer cells, suggesting an important role in host defense against cancer progression. Cytotoxicity mediated by tumor-infiltrating NK cells may be an important determinant of outcome in NSCLC. In common with other innate immune effectors, the antitumor activity of NK cells is mediated independent of antigen-presentation mechanisms. Direct lysis of malignant cells that have altered surface expression of MHC class I molecules occurs in the context of release of perforin, a cytolytic protein that inserts itself into the plasma membrane of the target cell.[84]

There is evidence that patients exposed to cigarette smoke, the commonest risk factor for the development of NSCLC, have attenuated circulating NK cell activity.[85] Similarly, bronchoalveolar lavage sampling of smokers reveals reduced NK functioning in comparison with nonsmokers.[86] Although capable of direct tumor cell lysis in in vitro models, there is emerging data that NK recognition and destruction of tumor cells in NSCLC is impaired. Esendagli et al.[87] recently showed reduced numbers of NK cells within cancer tissue compared with adjoining nonmalignant lung regions. Downregulated expression of several markers of NK activation (e.g., NKG2D, NKp46, NKp30, and NKp44) may account for the apparent reduced cytotoxicity within tumors. This finding may help explain the attenuation of NK-mediated direct cancer killing mechanisms. There is also emerging evidence that dampening of NK cell-associated innate immune surveillance is mediated in a TGF-β-dependent manner via crosstalk with local Tregs.[88]

The prognostic relevance of host NK responses in NSCLC has been the focus of attention in a number of studies. Kerr et al.[89] showed a marginal benefit among postoperative cases with a high prevalence of NK tumor infiltration. This association was confirmed in two studies examining different histologic NSCLC subtypes. Takanami et al.[90] demonstrated a significant relationship between NK cell count and outcome in resected adenocarcinoma, although NK cell infiltration was not an independent prognostic factor in multivariate analysis. Villegas et al.[91] demonstrated a positive correlation between postoperative survival and numbers of intratumoral CD57$^+$ NK cells in patients after resection of squamous cell cancer, particularly in stage IB disease. Overall, the impact of NK cell infiltration seems greatest in early-stage disease, suggesting that as tumors progress, NK antitumor effector mechanisms become overwhelmed. Interestingly, an earlier study examining a more heterogeneous patient cohort did not identify any link between extent of NK cell infiltration as assessed by cytofluorometry and outcome.[63] However, considerable methodological differences among these various studies may account for the divergence of results. Furthermore, analysis of activation status of NK cells in cancer islets shows increased expression of the inhibitory receptor NKG2A, indicating an inactivated phenotype, whereas their stromal counterparts are characterized by an immune activated status.[92] Thus, evaluation of the numbers of tumor-infiltrating NK cells in isolation may be insufficient to generate prognostic information.

Co-operation between infiltrating NK and alveolar macrophages may be necessary for effective killing of transformed cells,[93] yet simultaneous assessment for both cell types has infrequently been carried out in pathologic studies. The number of NK cells in direct contact with cancer cells may also be an important consideration. With respect to anatomic microlocalization, most NK cells reside within the peritumoral stroma and are not in direct contact with cancer cells.[94] This finding further highlights the importance of detailed characterization of immune effector populations and may help explain the functional aberrancy of NK cells in the malignant environment.[95]

Macrophages

Macrophages are ubiquitous immune cells that direct a number of vital physiological and host defense processes, including pathogen phagocytosis, regulation of inflammation, and tissue repair. TAMs comprise a major constituent of the leukocyte infiltrate characteristic of malignant disease and are present in abundance in both primary and metastatic lesions. Within tumors, TAMs interact with cancer cells to produce a rich source of cytokines and growth factors that shape the local microenvironment.[96]

In recent years, a wealth of evidence has emerged supporting the contention that macrophages play a key role in tumor progression by facilitating cancer cell proliferation, migration and metastasis, stimulating angiogenesis, and suppressing host antitumor immunity. Nevertheless, there is considerable debate regarding the prognostic relevance of TAMs in NSCLC.[96,97] Although some investigators have shown that increased number of TAMs confer a survival advantage, others have identified this pattern as a marker of poor outcome. This discrepancy is almost certainly related to differences in numbers evaluated, tumor stage, histology, size, and grade as well as the wide variation in techniques used to analyze patterns of macrophage infiltration. However, perhaps most crucially, researchers have often not taken the microanatomical localization into consideration.[98,99]

The importance of accurate assessment of regional infiltrative patterns was highlighted in a study that demonstrated that microanatomical distribution of macrophages in resected NSCLC specimens exerts a powerful impact on survival.[49] Specifically, macrophage infiltration

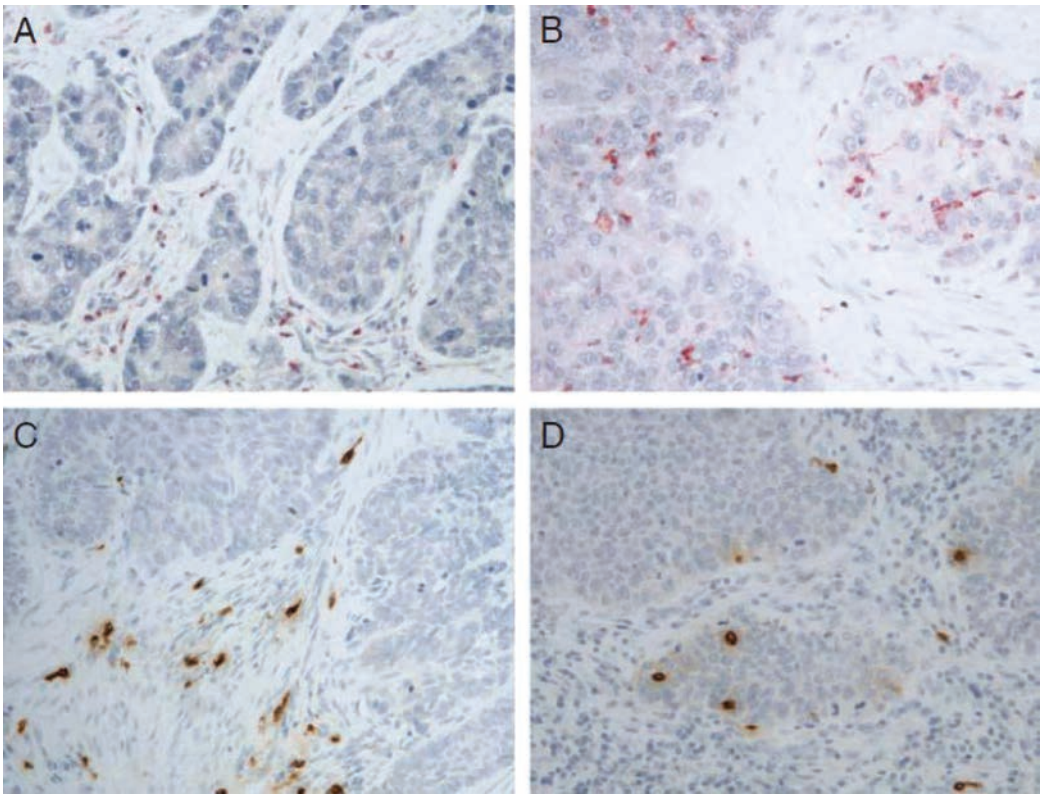

FIGURE 1. Immunohistochemical staining of non-small cell lung cancer tissue sections showing the presence of $CD68^+$ macrophages (red) in (*A*) tumor stroma and (*B*) tumor islets and of $tryptase^+$ mast cells (brown) in (*C*) tumor stroma and (*D*) tumor islets. Patients demonstrating immune cell infiltration patterns similar to panels (*B*) and (*D*) showed improved 5-year survival rates. Reprinted with permission from *J Clin Oncol* 2005;23:8959–8967.

of tumor cell islets was shown to confer a marked survival advantage, whereas stromal macrophages were associated with a significantly worse prognosis (Figure 1). Both increasing tumor cell islet macrophage density and islet/stromal macrophage ratio also emerged as favorable independent prognostic indicators. Stromal macrophage density was established as an independent predictor of poor survival. Notably, 5-year survival for patients with islet macrophage density above the median was 52.9 versus 7.7% with those below the median ($p < 0.0001$). Moreover, similar results were evident when patients were divided into those with a complete or incomplete surgical resection.

Functional plasticity is a well-described characteristic of macrophages of which there are at least two major classes, referred to as M1 ("classically activated") and M2 ("alternatively activated") macrophages.[100] While M1 macrophages elaborate proinflammatory cytokines and are regarded as having an antitumor phenotype, M2 macrophages are recruited into tumors and facilitate malignant progression through impairment of adaptive immunity. Recently published data seem to support the hypothesis that macrophages populating tumor islets represent a population that is characterized by a predominant M1 phenotype. Using immunohistochemistry to quantify expression of the cytotoxic markers TNF-α, HLA-DR, inducible nitric oxide synthase, and myeloid-related protein-8/14, Ohri et al.[101] showed that patients with prolonged survival after surgical resection show a predominance of tumor islet macrophages characterized by an M1 phenotype compared with those with poor survival.

Two subsequently published studies have reported consistent findings regarding the role of inflammatory infiltrate microlocalization, further supporting the notion that lung cancers are invaded by at least two distinct macrophage types. Using tissue microarray techniques, Kim et al.[102] showed that patients with a high tumor islet macrophage density survived longer after surgical resection compared with those with low tumor islet macrophage density, irrespective of stage (5-year survival rates, 63.9 versus 38.9%, respectively, $p = 0.0002$). By comparison, assessment of total macrophage count alone was not an independent predictor of outcome. High numbers of stromal macrophage were also associated with worse outcome within the high or low tumor islet macrophage groups. Patients with high tumor/low stromal macrophage counts survived longest, whereas those with low tumor islet/high stromal macrophage counts had shortest survival.

Kawai et al.[66] evaluated the prognostic impact of immune infiltrate patterns in patients with stage IV NSCLC treated with standard platinum-based doublet chemotherapy. These investigators similarly focused on the

role of inflammatory cell microlocalization using tumor biopsy specimen to study the relative distribution of macrophages, mast cells, and $CD8^+$ T-cells in the different tumor compartments. Results showed that individuals with higher tumor islet/stroma macrophage ratio had a median survival time more than double that of those with more macrophages in stroma than islets. The 1-year survival rates in the two groups were 60.8 versus 21.4%, respectively. Patients with a combined high tumor islet/stroma macrophage ratio and high tumor islet/stroma CD8+T ratio had an even greater survival advantage, and regional distribution of both cell types emerged as favorable independent prognostic factors. By contrast, an increased macrophage infiltrate in cancer stroma conferred a significantly worse outcome compared to low stroma macrophage counts.

Taken together, these studies indicate that macrophages located in tumor islets exhibit a predominantly antitumor phenotype, whereas those in adjacent stroma are characterized by proangiogenic, antiapoptotic effects that support cancer growth and dissemination. Indeed, TAMs populating tumor islets, particularly in patients with favorable outcomes, seem to be distinguished by a distinct "cytotoxic" phenotype.[101] By contrast, populations within the peritumoral stroma secrete cytokines that favor angiogenesis and tumor progression.[76] Such a hypothesis would help account for apparent contradictory results seen in different studies evaluating TAMs and outcome in NSCLC.[98,103]

The phenotype of macrophages is critically dependent on their differentiation and activation status. Using immunostaining, Zeni et al.[104] showed that the proportion of TAMs (but not tumor cells) expressing IL-10 is increased in late-stage NSCLC, was more frequently observed in those with lymph node metastases, and predicts worse overall survival. This suggests that these phagocytes behave as M2-polarized TAMs and are complicit in neoplastic extension by promoting angiogenesis, tissue remodeling, and suppression of protective CMI responses.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) represent a distinct population of immature myelomonocytic cells that act as potent suppressors of host innate and adaptive immunity. Expression of MDSCs is increased in the setting of a variety of clinical contexts, including bacterial and parasitic infections, autoimmunity, chemotherapy, and cancer.[105] MDSCs that accumulate at tumor sites represent a heterogeneous population consisting of granulocytes, monocytes/macrophages, and dendritic cells at various stages of differentiation that are characterized by the expression of the common human granulocyte and myeloid markers CD11b and CD33, respectively, but lack CD14 or HLA markers of mature myeloid lineage cells. As such, MDSCs do not represent a defined myeloid cell subset, and their phenotype is critically dependent on the tissue type they populate as well as the pattern of tumor-derived factors elaborated.

The cardinal feature of MDSCs is potent suppression of T-lymphocyte functionality. A number of inflammatory mediators produced either by cancer cells or others host immune elements that populate the tumor microenvironment are believed to drive expansion of MDSC numbers including COX2, prostaglandins, IL-1β, IL-6, granulocyte macrophage colony-stimulating factor, and vascular endothelial growth factor (VEGF), among others.[106,107] The subsequent activation of MDSCs is further dependent on several factors of which IFN-γ, IL-4, IL-13, and TGF-β are believed to be most important. The primary method of MDSC-associated immunosuppression is through interference with normal T-cell functioning via induction of the enzymes inducible nitric oxide synthase and arginase-1. In turn, nitration of the TCR prevents normal T-cell peptide-MHC interactions, resulting in failure to recognize and/or process specific tumor antigens.[108] In this fashion, MDSCs facilitate tumor immune evasion and cancer progression.

Most observations describing the immunosuppressive functionality exhibited by MDSCs have thus far been derived from animal studies, and data pertaining to the phenotype of this population in human NSCLC are lacking. Nevertheless, nearly a decade ago, Almand et al.[109] identified a population of circulating $CD34^+CD33^+CD15^-$ cells (comprising both immature macrophages and dendritic cells) capable of suppressing antigen-specific T-cell activity in the peripheral blood of NSCLC patients. Accumulating evidence now suggests that MDSCs are capable of promoting the de novo expansion of $Foxp3^+$ Tregs, thereby further contributing to the subversion of host antitumor immunosurveillance via indirect inhibition of effector T-cell activation and impairment of CMI. Whether this increase in Treg numbers is the result of conversion of Tregs from effector T lymphocytes or expansion of preexisting naturally occurring Treg numbers remains unclear.[110] The induction of Tregs is driven by increases in IFN-γ, IL-10, and/or TGF-β, depending on the nature of the myeloid precursor phenotype.[111] Although these various observations indicate that crosstalk between MDSCs and Tregs is important in contributing to $CD8^+$ T-cell anergy, it should also be noted that MDSCs are also capable of directly suppressing effector lymphocyte activity (i.e., in the absence of regulatory lymphocyte subsets), thereby facilitating tumor immune evasion.[110]

In addition to the various effects they exert on T-cell activation, MDSCs also impair innate inflammatory cell antitumour responses. In this regard, circulating and splenic-derived MDSCs may migrate to tumor sites and through direct cell-to-cell contact promote differentiation of macrophages to an M2, alternatively activated, tumor-promoting phenotype via an increase in IL-10 levels and a concomitant decrease in macrophage-derived IL-12 levels, further driving a predominant T_H2 response that is conducive to cancer cell proliferation.[112] These cells also seem to enhance tumor growth directly via additional nonimmune mechanisms, including promotion of angiogenesis, invasion, and metastasis.[113]

Given their central role in immune suppression and cancer progression, there is a strong rationale for the use of therapeutic strategies with the potential to modulate MDSC activity. Interestingly, gemcitabine, which is routinely used in NSCLC treatment regimens, has shown activity against MDSCs.[112] Approaches that limit MDSC intratumoral accumulation seem to be of particular interest in the context of cancer immunotherapy. However, the phenotypic heterogeneity that is the hallmark of MDSCs reduces the likelihood that the development of agents that act on a single signaling pathway would be effective in limiting induction of this population in cancer patients. Furthermore, caution must be exercised when attempting to extrapolate data derived from animal models of disease to human lung cancer. To the best of the current authors' knowledge, no studies specifically evaluating the clinical relevance of tumor islet versus stroma microlocalization with respect to MDSCs have been performed to date. As such, to realize a therapeutic alternative that targets MDSCs, an improved understanding of the complex interactions within the tumor microenvironment between tumor cells, MDSCs, and mature host immune populations, in particular Tregs, is necessary.

Mast Cells

Accumulation of mast cells is characteristic of a number of angiogenesis-dependent conditions including arthritis,[114] wound repair,[115] and cancer.[116–118] Mast cells have been shown to be a significant source of the proangiogenic molecules VEGF, TGF-β, and basic fibroblast growth factor, and there is evidence to suggest that mast cell infiltration may mediate angiogenesis in different tumor types.[119,120] The two major secretory products of mast cells are chymase and tryptase.

As is the case for other inflammatory cell elements, there are conflicting data regarding the relationship between mast cell infiltration and outcome in NSCLC, although most studies have tended to show that increased mast cell infiltration correlates with intratumoral angiogenesis and a poor prognosis. In lung adenocarcinoma for example, VEGF-positive tumors seem to induce intratumoral migration of mast cells. A strong association has also been demonstrated between increased tryptase-positive mast cell (MC_T) and tryptase- and chymase-positive mast cell (MC_{TC}) density and microvessel count at the advancing tumor edge in adenocarcinoma.[121] Imada et al.[122] demonstrated VEGF expression in stromal mast cells and showed that enhanced stromal mast cell infiltrate correlates with microvessel density in moderate-to-well differentiated adenocarcinoma. Mast cell counts were noted to increase as tumor stage increased. These authors suggested that new microvessel growth may be promoted in regions of mast cell accumulation, accounting for the worse prognosis observed in patients after surgical resection for stage I disease.

Mast cell density was shown to correlate with increased microvessel counts and was identified as an independent predictor of poor survival among 180 patients who underwent curative-intent surgical resection for pulmonary adenocarcinoma.[123] Tomita et al.[124] also showed a strong correlation between mast cell infiltration and angiogenesis in both adenocarcinoma and squamous cell carcinoma. Interestingly, these same investigators found that increased mast cell numbers correlated with improved survival among patients who underwent surgery for adenocarcinoma,[125] and tumors characterized by higher mast cell counts were more likely to be well differentiated. However, the precise intratumoral distribution of mast cells in these various studies was not reported.

It is important to note that a range of methodologies have been used to quantify mast cells and study their relationship to angiogenesis in NSCLC, with various criteria used to define high versus low inflammatory population infiltrates. Moreover, simple quantification of the absolute number of tumor-associated mast cells is not necessarily a reflection on the proportion of this population that are functionally active.[126] Perhaps crucially, patterns of mast cell microlocalization have infrequently been reported by researchers examining the prognostic relevance of mast cells in NSCLC, a fact that may explain the discordant results from previous studies. The importance of this methodological consideration was highlighted in work by Welsh et al., who evaluated surgical resection specimens from 175 patients. These investigators showed that a high tumor islet/stromal mast cell ratio independently predicts favorable outcome (Figure 1).[49] Patients with total islet mast cell counts above the median had an overall 5-year survival of 40%, compared with 22% for those with total mast cell counts below the median value. Thus, whether mast cells predominantly exert a pro- or antitumor effect in NSCLC likely depends on a number of different factors, including their precise microlocalization within the tumor, the predominant pattern of protease elaboration (i.e., MC_T versus MC_{TC}), and interactions with surrounding tumor cells and other host immune elements.

SUMMARY

The precise mechanisms that underlie the different patterns of inflammatory cell infiltration discussed in this review remain to be more clearly elucidated. Moreover, no standardized scheme exists to assess and quantify lymphoreticular infiltrates, leading to difficulties in comparisons between studies and the generalizability of results. Nonetheless, continued refinements in our understanding of the complex interplay between different components of the host response in NSCLC are vital to identify potential new treatment approaches for this devastating disease. Ultimately, speculative immunomodulatory strategies that preserve effective antitumor host immune response while simultaneously downregulating pro-tumor inflammatory cell populations are more likely to be translated into successful therapies in the clinical setting in future.

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