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The Biologic Importance of Tumor-Infiltrating Lymphocytes

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Abstract

Detailed pathologic analysis has delineated a close association between intra-tumoral $CD8^+$ cytotoxic T cells and favorable clinical outcomes in diverse cancers. Conversely, the presence at tumor sites of negative immune regulatory elements, such as $FoxP3^+$ T cells (Tregs) and PD-1/PD-L1 co-stimulatory molecules, is closely associated with inferior patient survival. Together, these results indicate the importance of the balance between cytotoxic and regulatory pathways in the tumor microenvironment as a critical determinant of prognosis. This immune index also provides a framework for devising therapeutic strategies to enlarge the population of anti-tumor cytotoxic T cells and attenuate immune regulation. Among these approaches, vaccination with irradiated, autologous tumor cells engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) followed by antibody blockade of cytotoxic T lymphocyte associated antigen-4 (CTLA-4) provides clinical benefits for some advanced-course melanoma patients. The extent of tumor necrosis in post-treatment biopsies is linearly related to the natural logarithm of the ratio of $CD8^+$ T cells to $FoxP3^+$ Tregs. These findings reveal a concordance between the immune signature of tumor protection in endogenous and therapy-induced responses, strongly supporting Martin Mihm's original insights.

Introduction

Dr. Martin Mihm's pioneering characterization of tumor-infiltrating lymphocytes in malignant melanoma has catalyzed research on the host response in the tumor microenvironment. Cancer cells may evoke recognition by both the innate and adaptive immune systems (1). The innate response, which involves granulocytes, macrophages, NK, NKT, and dendritic cells, is the first to be triggered and exploits germ-line encoded pattern recognition receptors to detect stress-induced molecules on tumor cells. The adaptive response, which involves $CD4^+$ and $CD8^+$ T lymphocytes and antibody-producing B cells, is slower to evolve but manifests long-term memory, reflecting the selection and expansion of rare lymphocytes that are specific for tumor-associated antigens.

Substantial evidence indicates that the immune system participates in cancer pathogenesis and may contribute to either disease progression or inhibition of tumor growth (2). These dual roles reflect the complex interplay of innate and adaptive immune elements with cancer cells and non-transformed stromal elements in the tumor microenvironment. These immune interactions resemble the dynamics of wounds that fail to heal, underscoring the oncogenic risks of recurrent tissue injury and abortive attempts at tissue repair (3). While the role of

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Conflicts of interest
To be confirmed.

smoldering inflammation in tumor promotion is discussed elsewhere (4, 5), here we highlight clinical evidence, inspired by Dr. Mihm's pioneering investigations, linking some anti-tumor immune responses with favorable patient outcomes.

Prognostic importance of intra-tumoral lymphocytes

There is compelling evidence that malignant melanoma can evoke immune responses in some patients. Drs. Martin Mihm and Wallace Clark first showed that the radial growth phase of primary melanoma typically elicits a significant dermal lymphocyte reaction that can effectuate partial tumor destruction (6). Clonal T cell expansions have been documented in primary regressing melanoma, and these lymphocytes manifest cytotoxicity towards autologous, cultured melanoma cells (7, 8). $CD4^+$ and $CD8^+$ T cells that react with melanoma cells can be detected in the blood, lymph nodes, and metastases of many patients (9). Moreover, in rare cases, widely disseminated melanoma may undergo spontaneous regression, accompanied by a diffuse infiltrate of lymphocytes, plasma cells, and macrophages (10).

Years ago, these findings prompted Drs. Clark and Mihm to explore the relationship between host responses to melanoma and survival. Through careful morphologic analysis, they established that dense intra-tumoral (but not peri-tumoral) T-cell infiltrates in the vertical growth phase of primary melanoma are tightly correlated with prolonged survival and a reduced incidence of metastatic disease (11, 12). Dr. Mihm and colleagues further showed that brisk T-cell reactions in melanomas that metastasize to regional lymph nodes are similarly predictors for improved survival when compared to lesions that fail to elicit infiltrates (13). These provocative results are currently being extended in several international cohort studies of thousands of patients with long-term clinical follow-up.

Dr. Mihm's pioneering investigations have motivated several other groups to explore a potential link between T-cell infiltrates and patient outcome. Remarkably similar correlations between tumor-associated T cell infiltrates and prolonged survival have been delineated in ovarian (14), colon (15–17), renal cell (18), and non-small cell lung carcinomas (19) as well as follicular lymphomas (20).

However, an important issue is why these host responses fail to prevent disease development. Multiple immunosuppressive mechanisms in the tumor microenvironment may restrain the breadth and magnitude of host cytotoxic reactions (2). Prominent among these are a small population of professional regulatory T cells characterized by expression of the fork-headed winged-helix transcription factor FoxP3 (21–25). These lymphocytes develop naturally in the thymus or may be generated in the periphery in the presence of non-inflammatory antigen exposure and TGF-β (26, 27).

$FoxP3^+$ regulatory T cells (Tregs) inhibit tumor-specific $CD8^+$ T-cell killing and restrict the effector functions of NK cells and CD1d-restricted NKT cells (28, 29); in murine models, depleting these cells enhances immune-mediated tumor destruction (30, 31). The clinical relevance of Treg-mediated immune suppression is illustrated by the shorter survival of patients harboring the largest numbers of intra-tumoral $FoxP3^+$ cells. Indeed, lymphocyte infiltrates rich in $CD8^+$ cytotoxic T cells but deficient in $FoxP3^+$ regulatory T cells are tightly correlated with improved patient outcomes following standard oncologic therapy, highlighting the balance of these subsets as a critical determinant of protective immunity (32–35).

Complementing these findings are recent investigations establishing a critical role for the PD-1/PD-L1 co-stimulatory pathway in restraining the action of anti-tumor cytotoxic T cells (36). The increased expression of PD-1 or PD-L1 on tumor and/or infiltrating T cells in

ovarian cancer (37), pancreatic cancer (38), renal carcinoma (39), and hepatocellular carcinoma (40) is linked with inferior survival. It will be of particular interest to determine whether PD-1/PD-L1 expression could further refine the prognostic value of the ratio of $CD8^+$ to $FoxP3^+$ T cells.

Therapy-induced anti-tumor immune responses

The analysis of endogenous host responses raises the possibility that therapeutic strategies that augment anti-tumor cytotoxic T cells and inhibit $FoxP3^+$ Tregs might potentiate protective immunity. Towards this end, we found that vaccination with irradiated GM-CSF-secreting tumor cells stimulated potent, specific, and long-term protective immunity in several murine tumor models (41). Immunization elicited a dense local infiltrate of macrophages, granulocytes, and $CD11b^+$ dendritic cells that expressed high levels of B7-1, B7-2, MHC class II, and CD1d (42). This reaction resulted in the efficient phagocytosis and processing of dying tumor by the elicited dendritic cells, which in turn migrated to the regional lymph nodes to stimulate tumor-specific lymphocytes. $CD4^+$ and $CD8^+$ T cells, CD1d-restricted invariant NKT cells, and antibodies mediated protective immunity (41–46).

These pre-clinical experiments provided the foundation for Phase I clinical trials of vaccination with irradiated, autologous melanoma cells engineered to secrete GM-CSF in stage IV metastatic melanoma patients (47, 48). Surgically excised tumors were processed to single cells, introduced into short-term culture, and transduced with a replication-defective retrovirus or adenovirus encoding GM-CSF (typically achieving three log increases in cytokine production). The tumors were then irradiated and cryopreserved. No serious toxicity was detected. Most patients developed strong local response to the vaccine characterized by dense infiltrates of dendritic cells, macrophages, eosinophils, and lymphocytes. Though metastatic lesions resected prior to vaccination contained minimal immune infiltrates, metastatic lesions resected following vaccination revealed dense infiltrates of $CD4^+$ and $CD8^+$ T lymphocytes and plasma cells with extensive tumor destruction (at least 80%), fibrosis, and edema in approximately two-thirds of the lesions examined. We also found targeted destruction of the tumor vasculature by activated lymphocytes, eosinophils, and neutrophils. T lymphocytes harvested from the necrotic lesions produced a broad range of cytokines and manifested cytotoxicity to autologous tumor cells. High titers of IgG antibodies reactive with melanoma determinants were identified by flow cytometry and western analysis. Although objective responses were uncommon, approximately 20% of immunized stage IV patients achieved long-term survival in excess of five years. Investigation of blood and tumor samples from vaccinated patients yielded multiple targets of immune-mediated tumor destruction (49).

Despite these reactions, most immunized patients eventually succumbed to progressive disease, indicating that additional defects remain to be addressed. Substantial evidence implicates a critical role for CTLA-4 in attenuating anti-tumor immunity (50). CD28 engagement by B7-1 or B7-2 provides an important co-stimulatory signal for effector T cells, but the subsequent triggering of CTLA-4 by these ligands decreases cytokine production and restrains cellular proliferation (51–53). Moreover, regulatory T cells constitutively express CTLA-4, which is required for their function (54, 55). Although young mice deficient in CTLA-4 develop lethal autoimmunity (56–58), Allison and colleagues showed that transient anti-CTLA-4 antibody blockade increased tumor immunity, with only a partial loss of tolerance to normal tissues (59). CTLA-4 inhibition, either as monotherapy or in conjunction with chemotherapy, provoked tumor regression in a variety of immunogenic models, while combinations with GM-CSF-secreting tumor cell vaccines resulted in synergistic effects in poorly immunogenic models (60–66). Therapeutic

immunity was associated with an expansion of $CD8^+$ cytotoxic T cells and inhibition of Tregs at tumor challenge sites (67).

Based on these pre-clinical studies, we administered a fully human anti-CTLA-4 mAb (Ipilumimab; 3 mg/kg at 2–3 month intervals) to 14 metastatic melanoma patients who were previously immunized with irradiated, autologous, GM-CSF-secreting tumor cells (beginning one month after vaccination) (68, 69). Inflammatory toxicities were restricted to a single case of grade-II colitis, a single case of asymptomatic hilar adenopathy (resembling sarcoidosis) that resolved without therapy, and 13 cases of grade I-II skin rashes. Notwithstanding the absence of grade III-IV toxicities, 10 patients achieved clinically meaningful anti-tumor effects, which included four partial responses (53+, 43+, 41+, 20 months) and six stable diseases (46+, 45+, 18, 11, 9, 6 months). Pathologic examination of metastases resected following therapy revealed $CD4^+$ and $CD8^+$ T cells, $CD20^+$ immunoglobulin-producing B cells, and granulocytes juxtaposed to dying melanoma cells. Quantitative analysis of intra-tumoral infiltrates showed that the extent of tumor necrosis was linearly related to the natural logarithm of the ratio of $CD8^+$ T cells to $FoxP3^+$ Tregs, suggesting that the balance between these subsets may be a critical determinant of therapeutic effect (Figure 1).

Conclusions

Dr. Martin Mihm's careful morphologic examination of early-stage melanomas first highlighted the prognostic importance of tumor-infiltrating lymphocytes. Further detailed studies of the tumor microenvironment have begun to unravel the major pathways that dictate the outcome of the anti-tumor immune response. The balance of cytotoxic and regulatory pathways has emerged as a major index of tumor destruction in both endogenous and therapy-induced reactions. Additional histologic, immune, and genetic characterization of tumor-infiltrating lymphocytes is likely to prove instrumental to the development of clinically efficacious immunotherapies.

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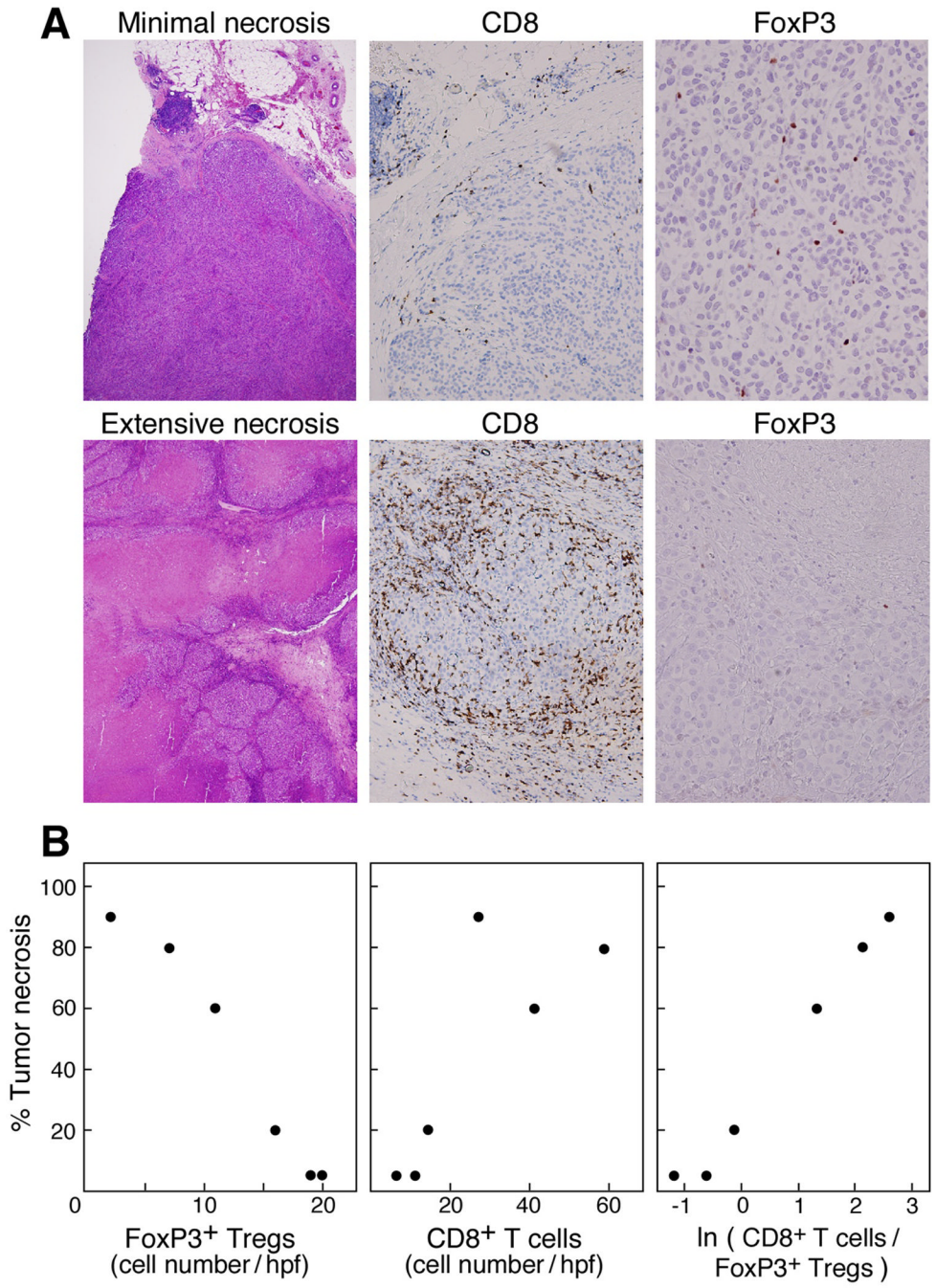

Figure 1.

The ratio of tumor-infiltrating $CD8^+$ T cells to $FoxP3^+$ Tregs following administration of GM-CSF-secreting melanoma vaccines and anti-CTLA-4 antibody infusion is tightly correlated with the extent of tumor necrosis in advanced melanoma patients. (A). Top, minimal necrosis of melanoma metastasis; bottom, extensive necrosis of melanoma metastasis. (Magnification: H&E ×4; CD8, ×20; FoxP3, ×40.) (B). Numbers of intra-tumoral $FoxP3^+$ Tregs and $CD8^+$ T cells versus tumor necrosis. Reprinted from (69)