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Human tumor-associated monocytes/macrophages and their regulation of T cell responses in early-stage lung cancer

Sunil Singhal¹, Jason Stadanlick¹, Michael J. Annunziata¹, Abhishek S. Rao¹, Pratik S. Bhojnagarwala¹, Shaun O'Brien², Edmund K. Moon², Edward Cantu³, Gwenn Danet-Desnoyers⁴, Hyun-Jeong Ra⁴, Leslie Litzky⁵, Tatiana Akimova^{5,6}, Ulf H. Beier⁷, Wayne W. Hancock^{5,6}, Steven M. Albelda², Evgeniy B. Eruslanov^{1,*}

¹Division of Thoracic Surgery, Department of Surgery, University of Pennsylvania, Philadelphia, PA 19104, USA.

²Division of Pulmonary, Allergy, and Critical Care, University of Pennsylvania, Philadelphia, PA 19104, USA.

³Division of Cardiovascular Surgery, Department of Surgery, University of Pennsylvania, Philadelphia, PA 19104, USA.

⁴Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

⁵Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

⁶Division of Transplantation Immunology, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA.

⁷Division of Nephrology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA.

Abstract

Data from mouse tumor models suggest that tumor-associated monocyte/macrophage lineage cells (MMLCs) dampen antitumor immune responses. However, given the fundamental differences between mice and humans in tumor evolution, genetic heterogeneity, and immunity, the function of MMLCs might be different in human tumors, especially during early stages of disease. Here, we studied MMLCs in early-stage human lung tumors and found that they consist of a mixture of

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*Corresponding author. evgeniy.eruslanov@uphs.upenn.edu.

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classical tissue monocytes and tumor-associated macrophages (TAMs). The TAMs coexpressed M1/M2 markers, as well as T cell coinhibitory and costimulatory receptors. Functionally, TAMs did not primarily suppress tumor-specific effector T cell responses, whereas tumor monocytes tended to be more T cell inhibitory. TAMs expressing relevant MHC class I/tumor peptide complexes were able to activate cognate effector T cells. Mechanistically, programmed death-ligand 1 (PD-L1) expressed on bystander TAMs, as opposed to PD-L1 expressed on tumor cells, did not inhibit interactions between tumor-specific T cells and tumor targets. TAM-derived PD-L1 exerted a regulatory role only during the interaction of TAMs presenting relevant peptides with cognate effector T cells and thus may limit excessive activation of T cells and protect TAMs from killing by these T cells. These results suggest that the function of TAMs as primarily immunosuppressive cells might not fully apply to early-stage human lung cancer and might explain why some patients with strong PD-L1 positivity fail to respond to PD-L1 therapy.

INTRODUCTION

Immunotherapies directed toward boosting host antitumor immunity are at the forefront of cancer therapeutics. However, despite recent successes with checkpoint blockade and adoptive T cell transfer, these immunotherapies often fail to induce a durable antitumor response in solid tumors in a substantial percentage of patients with cancer (1, 2). This lack of efficacy suggests that a deeper understanding of the interactions of tumor-specific T cells with other immune cells within human tumor microenvironment is necessary to improve cancer immunotherapy.

Monocyte/macrophage lineage cells (MMLCs) accumulate in many types of human and murine tumors and are thought to regulate nearly every step of tumor development, including antitumor T cell responses (3, 4). Our current understanding of tumor-associated MMLCs is based primarily on studies performed in murine transplantable tumor models. In these murine studies, tumor-infiltrating MMLCs are largely comprised of macrophages and monocytic myeloid-derived suppressor cells (Mo-MDSCs) that exert a predominantly protumoral and immunosuppressive role in cancer development (5, 6). However, the antitumor function of MMLCs, including the augmentation of adaptive immune responses, has also been reported (7–10). Note that most of the transplantable mouse tumor models use tumor cell lines originally derived from advanced tumors that have already been subjected to immune selection and thus grow rapidly in vivo (11). Accordingly, these mouse models lack prolonged initial phases of multistage tumor evolution and, for the most part, reflect the immune response as it exists during advanced stages of tumor development at which time protumoral mechanisms already prevail. In contrast, human tumors evolve much more slowly, with prolonged early stages of development in which sustained selective pressure by the host antitumor immune response appear to occur (12). Despite these differences, the function of MMLCs within early-stage human tumors remains unexplored.

Correlations of macrophage counts in surgical specimens with clinical prognosis have shown conflicting results regarding the prognostic role of tumor-associated macrophage (TAM) infiltration in different cancer types, including lung cancers (LCs) (13). Thus, there is still a general lack of consensus regarding the prognostic role of TAM density in patients with LC

(14, 15), and these studies can only provide correlative, but not causative, links between TAMs and disease progression.

TAM functions now have direct clinical relevance with the success of anti-programmed death-ligand 1 (anti-PD-L1) monoclonal antibody (mAb) therapy in a subset of patients with LC (1, 16). However, the precise mechanisms underlying the clinical response to this therapy are still not well understood. It appears that tumor-expressed PD-L1 plays a direct T cell inhibitory role, but the significance of PD-L1 expressed by MMLCs in the regulation of the effector phase of tumor-specific T cells remains controversial in mouse models and is unclear in humans (17, 18).

Given this knowledge gap in our understanding of the biology of MMLCs in early-stage human lung tumors, where tumor-induced immunosuppressive mechanisms might not be fully developed, the goals of this study were to (i) determine the phenotypic composition of tumor-infiltrating MMLCs, (ii) assess how different cell populations of this lineage regulated the effector phase of tumor-specific T cell responses, and (iii) explore the role of PD-L1 expressed on tumor-associated MMLCs or tumor cells in the regulation of tumor-specific T cell responses.

RESULTS

Tumor-associated MMLCs consist of a mixture of classical monocytes and macrophages coexpressing M1 and M2 markers

To date, deep immune profiling of human lung tumors has been mostly at the molecular level and is based on transcriptomic data interpreted by computational analysis (19–21). Although this approach is informative, it does not allow simultaneous assessment of the functional roles of identified cell phenotypes. Therefore, we performed phenotypic profiling of early-stage MMLCs at the cellular level to study the functions of cells with defined phenotypes.

To assess the phenotype of MMLCs in human LC, we analyzed cells from three locations: LC tissue, adjacent lung parenchyma (within the same lobe and termed “distant tissue”), and peripheral blood from patients with cancer and healthy donors (HDs). As additional controls, we analyzed MMLCs from noncancerous lung tissue obtained from potential donor lungs that were rejected for transplantation. We performed phenotypic analyses on resected samples from 93 patients with stage 1 and 2 non-small cell lung cancer (NSCLC). Detailed characteristics of all patients are shown in table S1.

We used an optimized approach to disaggregate human lung tumors, which minimizes enzyme-induced ex vivo effects on the viability, premature activation, phenotype, and function of human myeloid cells (22–24). Our phenotypic analyses of digested tumors showed that tumor-infiltrating CD45⁺CD11b⁺ myeloid cells (fig. S1A) were mostly composed of CD14⁺ MMLCs and CD66b⁺ granulocytic lineage cells (Fig. 1A). The total frequency of CD11b⁺ CD14⁺CD66b[−] cells in tumor digests varied from 1 to 19% of all live cells in the tumor microenvironment (Fig. 1B). The number of CD14⁺ cells in tumor tissue was significantly ($P = 0.001$) lower than in distant and noncancerous lung tissue, suggesting

that patients with early-stage LC did not actively accumulate MMLCs within their tumors. Comparative analysis of major monocyte chemotactic proteins (CC, CXC, and CX3C chemokine ligands CCL2, CCL7, CCL5, CCL3, CCL1, CX3CL1, and CXCL1) in conditioned medium collected from total tumor and distant lung tissue digests revealed that tumors significantly differed from distant tissue only by reduced secretion of CCL2 ($P = 0.04$; fig. S2A). We also found that the proportion of dead CD14⁺ cells was significantly ($P = 0.009$) elevated in the tumor tissue compared to the distant lung (fig. S2B). Therefore, decreased production of CCL2 and increased CD14⁺ cell death within the tumor might explain the lower number of CD14⁺ cells in tumors relative to distant lung tissue. There were no significant associations between the frequency of intratumoral CD11b⁺CD14⁺ cells in tumors and clinical parameters including tumor histologic type, size, stage, or smoking history (table S2).

Cytospins prepared from bead-sorted, tumor-infiltrating CD14⁺ cells revealed a heterogeneous morphology with cells resembling both macrophages and monocytes (Fig. 1A). Given these data, we investigated whether tumor-infiltrating CD11b⁺CD14⁺ cells were phenotypically different from their counterparts in distant tissue or noncancerous lung tissue. We found that the proportion of CD14⁺ cells coexpressing classical macrophage-associated markers was significantly higher in tumors than in distant tissues ($P < 0.01$; Fig. 1,C and D). Histological review of lung tumors stained for the commonly used macrophage marker CD68 showed that CD68⁺ cells were scattered throughout lung tumors but mostly infiltrated tumor stroma rather than tumor islets (Fig. 1E).

The progression of many types of human tumors, especially at advanced stages of disease, is accompanied by the accumulation of monocytic myeloid-derived suppressor CD14⁺ cells (Mo-MDSCs) with low or no expression of human leukocyte antigen–DR isotype (HLA-DR) in blood and tumor (25). Therefore, we determined whether the population of CD14⁺ MMLCs in early-stage tumors included these Mo-MDSCs. We found that tumors, distant tissues, and blood CD14⁺ cells expressed the HLA-DR molecule at three distinguishable intensities: high, intermediate, and low (Fig. 1F). The frequency of CD14⁺ cells with low or no expression of HLA-DR in the blood of patients with early-stage cancer was low (~6%) and not significantly different from their frequency in HDs (Fig. 1G). Interestingly, the frequency of HLA-DR^{lo/-}CD14⁺ cells was significantly lower in tumor tissues compared with blood of studied patients ($P < 0.001$; Fig. 1G and fig. S1B).

In contrast to HLA-DR^{lo/-}CD14⁺ cells, there was a remarkable accumulation of HLA-DR^{hi}CD14⁺ cells in tumors as compared to blood and distant tissue in most of the patients (Fig. 1, F and H, and fig. S1B). Cytospins prepared from flow-sorted tumor-infiltrating HLA-DR^{hi} and HLA-DR^{int}CD11b⁺CD14⁺ cells showed that HLA-DR^{hi}CD14⁺ cells typically demonstrated the morphology of macrophages, whereas HLA-DR^{int}CD14⁺ cells resembled monocytes (Fig. 1I). Further flow cytometric analysis of HLA-DR^{hi}CD14⁺ cells in tumor and distant lung tissue revealed that the macrophage-associated markers were highly expressed almost exclusively on HLA-DR^{hi}CD14⁺ cells (Fig. 2A, and B, and fig. S3).

On the basis of these data, we defined HLA-DR^{hi}CD14⁺ cells as TAMs and HLA-DR^{int}CD14⁺ cells as tissue/tumor monocytes (T-Mos) that could also include monocytes

from tumor vasculature. The vast majority of these T-Mos are represented by the classical CD14^{hi}CD16⁻ phenotype (fig. S4, A and B). The proportion of TAMs varied from 15 to 90% of all CD14⁺ cells in tumors (Fig. 2C) and from 0.1 to 9% among all cells in tumor digests (Fig. 2D). The frequency of macrophages within tumor-infiltrating CD14⁺ cells and total tumor digest was significantly higher compared to distant and noncancerous lung tissue ($P < 0.001$; Fig. 2C, and D). This increased accumulation of TAMs was associated with higher production of factors involved in the regulation of macrophage differentiation [granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage migration inhibitory factor (MIF), and monokine induced by IFN- γ (MIG)] in tumors compared to distant lung tissue (fig. S5A), but no differences in macrophage colony-stimulating factor (M-CSF). Our correlation analyses revealed no significant associations between the expression of macrophage-associated markers in tumors and key clinical parameters of patients with LC, including tumor histologic type, size, stage, or smoking history (table S2).

To assess the extent of human TAM phenotypic polarization, we compared the ex vivo phenotype of TAMs in tumor digests with phenotypes of classical human monocyte-derived M1 and M2 macrophages that were differentiated in vitro. Although monocyte-derived M1 and M2 macrophages showed clear differences in expression of respective markers, TAMs simultaneously expressed both M2 and M1 markers, and sometimes to an even higher degree than in vitro-differentiated M2 and M1 macrophages (Fig. 2E). Hence, the phenotype of macrophages in early-stage tumors is “mixed” and not predominately biased toward either M1 or M2 classical macrophage phenotypes. A heat map depicting the relative expression of macrophage markers among the CD14⁺ cells within tumors shows that CD14⁺ cells can be visually clustered into TAM-enriched tumors and T-Mo-enriched tumors based on the intensity of macrophage-associated marker expression (Fig. 2F). T-Mo-enriched tumors mostly accumulate a “classical” CD14^{hi}CD16⁻ monocyte subtype (fig. S4B).

To assess why certain tumors accumulated TAMs versus T-Mos, we incubated tumor digests overnight and compared the secretion of major factors involved in the regulation of macrophage differentiation and polarization and chemokines controlling monocyte recruitment. We found that digests from TAM-enriched tumors secreted significantly higher amounts of granulocyte-colony stimulating factor (G-CSF), whereas T-Mo-enriched tumors showed increased production of CCL3 ($P = 0.01$; fig. S5C). There was no difference in TAM and T-Mo viability in both types of tumors (fig. S5B).

Early-stage TAMs coexpress T cell coinhibitory and costimulatory receptors

Further flow cytometric analysis revealed that, in contrast to peripheral blood CD14⁺ monocytes, tumor- and distant lung tissue-infiltrating CD14⁺ cells markedly up-regulated both T cell coinhibitory and costimulatory molecules (Fig. 3A and figs. S3 and S6A). Although the expression of these molecules was highly heterogeneous, tumor-infiltrating CD14⁺ cells consistently exhibited high expression of the T cell coinhibitory and costimulatory molecules compared to their counterparts in distant and noncancerous lung tissue (Fig. 3A and fig. S6, A and B). Given the clinical importance of checkpoint blockade, we evaluated the PD-L1 surface expression in early-stage lung tumors. As anticipated, our histological review of lung tumors revealed that PD-L1 was expressed in both tumor islets

and tumor stroma (Fig. 3B). However, flow cytometric analysis of tumor digests demonstrated that surface PD-L1 expression on CD14⁺ cells was consistently higher than other cells in tumor digests, including malignant epithelial cell adhesion molecule (EpCam)⁺ cells (Fig. 3, C and D). Despite the heterogeneity, the proportion PD-L1^{hi}CD14⁺ cells among total CD14 cells, as well as total frequency of PD-L1^{hi}CD14⁺ cells among all nucleated cells, was significantly elevated in the tumor tissues of patients with LC compared to the distant and noncancerous lung tissue ($P < 0.001$; Fig. 3A and fig. S6C). The high PD-L1 surface expression in tumors was significantly associated with HLA-DR^{hi} CD14⁺ TAMs ($P = 0.0001$; Fig. 3E, and F). The proportion of these PD-L1^{hi}HLA-DR^{hi} TAMs among all CD14⁺ cells was statistically higher in tumors compared to distant tissue (Fig. 3G). We found that the PD-L1 surface expression significantly ($P < 0.05$) correlated with the intensity of T cell costimulatory molecule expression on tumor CD14 cells. Specifically, CD40, CD86, CD80, CD54, OX-40 L, and 4-1BBL (Fig. 3H and fig. S6D) were also mainly expressed by HLA-DR^{hi} CD14⁺ TAMs (fig. S3). When compared to noncancerous lung tissue, distant lung tissues had significantly ($P = 0.04$) increased accumulation of HLA-DR^{hi}CD14⁺ cells expressing both costimulatory and coinhibitory receptors, suggesting that tumors may affect activation and differentiation of macrophages in adjacent lung tissue.

A heat map depicting the relative expression of the classical T cell costimulatory and coinhibitory markers of the B7 family among CD14⁺ cells within tumors shows that intratumoral CD14⁺ cells could again be visually clustered into two groups (Fig. 3I). Although the proportion of these cells varied between patients, there was no accumulation of cells expressing predominantly either costimulatory or coinhibitory molecules in tumors (Fig. 3I). In addition, there were no significant associations between the extent of T cell costimulatory and coinhibitory molecule expression on TAMs and tumor stage, size, histologic type, or smoking history (table S2). Together, our phenotypic analysis demonstrates that the early-stage lung tumor microenvironment is primarily infiltrated with TAMs that have receptors with the potential to both stimulate and inhibit T cell responses.

Tumor-associated MMLCs exert diverse effects on antigen-nonspecific autologous T cell responses

A major challenge in human tumor immunology is deciphering the interaction of MMLCs with tumor-infiltrating T cells in the tumor microenvironment. To address this issue, we first performed commonly used antigen-nonspecific assays in which we determined the ability of tumor-derived CD14⁺ cells to alter autologous T cell responses after stimulation of PBMCs with plate-bound anti-CD3 mAbs (Fig. 4A, to D). Although we could clearly identify a relatively small population of patients whose tumor-derived CD14⁺ cells could inhibit IFN- γ production by T cells [Fig. 4A, (red box) and B] and T cell proliferation (Fig. 4D), we found that tumor CD14⁺ cells from majority of the patients were not primarily T cell suppressive; instead, they had various effects on IFN- γ production by T cells (Fig. 4A, and B) and T cell proliferation (Fig. 4C, and D). Most of the tumor-derived CD14⁺ cells had minimal or even stimulatory effects on T cell responses [Fig. 4A, (blue and green boxes) to D].

Next, we determined the effect of tumor and distant lung tissue CD14⁺ cells on IFN- γ production by CD8⁺ T cells from tumors or distant lung tissue by stimulating CD14-

depleted digests with plate-bound anti-CD3 antibodies (Abs) for 24 hours. Again, we observed that IFN- γ production in tumor-infiltrating CD8⁺ cells in most patients was unchanged or markedly reduced in the absence of tumor-associated CD14⁺ cells, suggesting a positive accessory role in this depletion model (Fig. 4, E and F). We could not find a significant correlation between the effects of CD14⁺ cells and patient clinical parameters, including tumor size, stage, type, or smoking history (table S2).

Tumor-associated MMLCs exert diverse effects on the effector phase of tumor-specific T cell responses

The antigen-nonspecific responses of resting T cells in PBMCs may not faithfully reflect events in tumors that are thought to be mostly infiltrated with antigen-specific effector or memory T cells (26, 27). Therefore, we investigated the effect of tumor-infiltrating CD14⁺ cells on the effector phase of tumor-specific T cell responses using a recently developed in vitro model (28). We transduced human T cells with a high-affinity transgenic T cell receptor (TCR) called Ly95 that recognizes a HLA-A*0201–restricted peptide sequence in the human testis cancer antigen New York esophageal squamous cell carcinoma 1 (NY-ESO-1) and genetically modified A549 human lung adenocarcinoma (AC) cells to express the NY-ESO-1 protein in the context of HLA-A*0201 (A549/A2–NY-ESO-1 cells) (28). Incubating Ly95 T cells with A549/A2–NY-ESO-1 tumor cells, but not with a control nontransduced A549 cell line, resulted in the acquisition of an activated phenotype (loss of CD62L and up-regulation of PD-1) by Ly95 T cells and robust production of IFN- γ and granzyme B in Ly95 T cells (Fig. 5A).

Before studying the effect of tumor-derived CD14⁺ cells on the tumor-specific T cell response, we first established the effects of non-tumor CD14⁺ cells from noncancerous lungs and CD14⁺ macrophages differentiated from monocytes of HDs in vitro as a baseline. For this purpose, we added these CD14⁺ cells as “third-party cells” to the NY-ESO-1–specific Ly95 T cells that were cultured with A549/A2–NY-ESO-1 target tumor cells and measured the IFN- γ production by Ly95 T cells at 24 hours. We found some diversity in the effects of CD14⁺ cells, allowing us to define a “normal” range due to donor-dependent variations in Ly95 cell responses (Fig. 5C, right columns). We then analyzed the functional effect of the tumor-derived CD14⁺ cells on Ly95 T cells in a large cohort of patients with early-stage LC (Fig. 5B, and C). Using our normal range of effects, all analyzed patients were divided into three groups: those whose tumor CD14⁺ cells suppressed Ly95 cells, those whose tumor CD14⁺ cells stimulated Ly95 cells, and those whose tumor CD14⁺ cells exerted a neutral effect [Fig. 5B, and C (red, green, and blue boxes)]. Most of the tumor CD14⁺ cells were within the HD-dependent range of CD14⁺ cell effects and thus showed minimal or no tumor-specific effects on Ly95 cell response (Fig. 5C, blue box). A matched-pair analysis revealed that CD14⁺ cells from distant tissue, in most cases, mirrored the effect of CD14⁺ cells from tumor, suggesting again that tumors affect the functional state of myeloid cells in adjacent tissues (Fig. 5C). The addition of tumor CD14⁺ cells to Ly95 T cells cocultured with control A549 parental tumor cells did not induce production of IFN- γ and granzyme B, indicating that the diverse effects of CD14⁺ cells on Ly95 cells were mostly NY-ESO-1–mediated and not the result of allostimulation (fig. S7, A and B). Direct cell-to-cell contact among tumor CD14⁺ cells, Ly95 cells, and target cells was required to observe these diverse CD14⁺

effects, because coculturing the Ly95 cells and A549/A2–NY-ESO-1 tumor cells in the presence of conditioned media collected from 24-hour tumor CD14⁺ cell cultures had no effect on Ly95 T cell response (fig. S7C). In addition, there was no effect of tumor CD14 cells on Ly95 cell responses when these cells were separated in the Transwell system (fig. S7C).

It is likely that, after tumor entry, tumor-specific effector T cells would first encounter and interact with stromal-based nonmalignant inflammatory cells, including CD14⁺ cells, before reaching their targets. To model this scenario, Ly95 cells were preincubated with tumor-isolated CD14⁺ cells for 24 hours before adding A549/A2–NY-ESO-1 tumor cells to cultures. In these experiments, tumor CD14⁺ cells did not modify the ability of Ly95 cells to produce IFN- γ in response to stimulation with A549/A2–NY-ESO-1 tumor cells at different ratios (Fig. 5D). These preincubated Ly95 cells were able to respond to A549/A2–NY-ESO-1 tumor cells to the same degree as when all cells were mixed simultaneously. Also, CD14⁺ cells isolated from tumor, distant tissue, and blood of patients with LC did not affect the activation phenotype of Ly95 cells that were preincubated with tumor-isolated CD14⁺ cells before stimulation (fig. S7D).

We did not find a significant correlation between CD14⁺ cell effects on tumor-specific T cells with any patient clinical parameters, including tumor size, stage, histologic type, or smoking history (table S2). There was no correlation between CD14⁺ cell-mediated T cell suppressive effects and the degree of any M2 macrophage marker expression (table S2) or the presence of tumor HAL-DR^{lo/-}CD14⁺ cells exhibiting a phenotype of Mo-MDSCs (fig. S7E).

To determine whether those rare tumor CD14⁺ cells that were able to suppress Ly95 T cell responses deployed common T cell suppressive pathways, we compared the gene expression of inducible nitric oxide synthases (*iNOS*), arginase-1 (*Arg-1*), indoleamine 2,3-dioxygenase (*IDO*), transforming growth factor- β (*TGF- β*), cyclooxygenase-2 (*COX-2*), interleukin-10 (*IL-10*), and nicotinamide adenine dinucleotide phosphate (*NADPH*) oxidase and the amount of secreted inhibitory factors and metabolites prostaglandin (PGE₂), IL-10, nitric oxide (NO), adenosine, arginine, l-lactate, and kynurenine in CD14⁺ cells isolated from tumor, distant tissue, and blood (figs. S8 and S9). Among all those pathways, we found that IDO/kynurenine, COX-2/PGE₂, and IL-10 were significantly up-regulated in tumor CD14⁺ MMLCs ($P < 0.05$; fig. S8A and S9A) compared to circulating CD14⁺ monocytes. However, there was no significant association between the degree of activation of these pathways in tumor CD14⁺ cells and the regulation of Ly95 cell responses [figs. S8 (B and C) and S9 (B and C)]. Under our conditions, we did not detect any iNOS and Arg-1 activity in tumor MMLCs by measuring mRNA or their metabolite production. The existence and role of these pathways in human macrophages remain disputed with regard to T cell suppression (29, 30).

Next, we measured the amount of PGE₂, IL-10, kynurenine, adenosine, and l-lactate in 24-hour cell culture supernatants collected from cell cocultures of Ly95 T cells, A549/A2–NY-ESO-1 tumor cells, and tumor CD14⁺ cells. Our comparative analysis revealed that suppression of Ly95 cells was not accompanied with significantly increased production of

those factors (fig. S10A). In addition, we demonstrated that the addition of inhibitors of IDO (Epacadostat), Arg-1 (CB-1158), reactive nitrogen (l-NMMA), or reactive oxygen species (apocynin) into this three-component system did not significantly change the magnitude of the Ly95 T cell response to targets in the presence of early-stage tumor MMLCs (fig. S10B).

To ensure that these diverse effects of CD14⁺ cells on antitumor T cells were not only limited to modulating IFN- γ production in Ly95 cells, we also assessed tumoricidal activity. Similarly, we found heterogeneous modulation of Ly95-mediated cell killing activity by tumor and distant CD14⁺ cells, whereas CD14⁺ cells isolated from blood had minimal effects (Fig. 5E, and F).

Last, we questioned whether tumor CD14⁺ cells could directly trigger NY-ESO-1–specific responses of Ly95 cells. We pulsed HLA-A2⁺ tumor-infiltrating CD14⁺ cells with NY-ESO-1_{157–165} peptide and cocultured the peptide-loaded tumor CD14⁺ cells with Ly95 T cells for 24 hours. In contrast to diverse effects of bystander tumor CD14⁺ cells on the effector phase of T cell responses, we found that all tumor CD14⁺ cells expressing HLA-A2/NY-ESO-1_{157–165} peptide complex were able to trigger IFN- γ production in Ly95 T cells (Fig. 5G, and H).

Unlike tumor cell–expressed PD-L1, PD-L1 on TAMs do not affect the interaction between tumor-specific effector T cells and targets

To directly assess the role of tumor-derived PD-L1 in the regulation of the effector phase of tumor-specific T cell responses, we coincubated NY-ESO-1–specific Ly95 T cells with A549/A2–NY-ESO-1 tumor cells genetically modified to express PD-L1. We observed that coculturing Ly95 T cells with PD-L1–negative (PD-L1[−]) A549/A2–NY-ESO-1 tumor cells resulted in robust production of IFN- γ and granzyme B in the Ly95 T cells, as well as tumor cell death (Fig. 6A). In contrast, expression of PD-L1 on A549/A2–NY-ESO-1 tumor cells markedly inhibited Ly95 cell activation (fig. S7D), killing activity and production of IFN- γ (Fig. 6A), although the granzyme B expression in Ly95 cells was not affected. Adding anti-PD-1 blocking Abs to the cocultures largely restored the Ly95 cell responses, confirming a mostly PD-L1–dependent effect (Fig. 6B).

Next, we explored the effect of MMLC-expressed PD-L1 on the NY-ESO-1–specific T cell responses, using a three-component system in which Ly95 T cells were stimulated with PD-L1[−] A549/A2–NY-ESO-1 tumor cells in the presence of “third-party” bystander tumor CD14⁺ cells that exhibited the natural variation of PD-L1 expression (Fig. 6C). Tumor-infiltrating CD14⁺ cells containing a high frequency of PD-L1 positive cells did not negatively regulate Ly95 T cell responses in this three-component system (Fig. 6C, top), whereas, as above, PD-L1–positive (PD-L1⁺) A549/A2–NY-ESO-1 tumor cells strongly inhibited Ly95 cell responses in the two-component system (Fig. 6C). The addition of PD-L1–blocking Abs into the three-component system did not affect the Ly95 cell response. In some cases, tumor CD14⁺ cell populations with a high proportion of PD-L1^{hi}HLA-DR^{hi} cells were capable of stimulating Ly95 T cell responses (SI >1; Fig. 6C, and D). In contrast, populations of CD14⁺ cells with low frequencies of PD-L1⁺ cells tended to have neutral or negative effects on Ly95 response [Fig. 6C, (bottom) and D]. A correlation analysis revealed that high Ly95 T cell stimulatory activity of CD14⁺ cells was associated with HLA-DR^{hi}PD-

L1^{hi} TAM-enriched tumors, whereas suppressive and neutral activity of CD14⁺ cells was mostly observed in monocyte-enriched tumors with relatively lower T cell coinhibitory and costimulatory molecule expression (Fig. 6E).

To more explicitly interrogate the role of PD-L1 on CD14⁺ cells in our three-component system, we isolated PD-L1^{hi} and PD-L1⁻ tumor-infiltrating CD14⁺ subsets from the same patients by flow cytometry cell sorting (Fig. 7A). Each sorted CD14⁺ subset was mixed with Ly95 T cells and A549/A2-NY-ESO-1 tumor cells, and this triad of cells was cultured for 18 hours. Ly95 cells cultured with PD-L1⁻ or PD-L1⁺ A549/A2-NY-ESO-1 tumor cells (without patient CD14⁺ cells) served as controls. Despite high PD-L1 expression, HLA-DR^{hi}PD-L1^{hi}CD14⁺ cells demonstrated various effects on effector responses to target cells, as measured by IFN- γ production in Ly95 cells (Fig. 7B, and C, and fig. S11, A and B). HLA-DR^{hi}PD-L1^{hi} CD14⁺ TAMs isolated from majority of the patients had a stimulatory or no effect [Fig. 7B, (red box) and C], with only a minority being able to distinctly suppress Ly95 cell responses (Fig. 7C and fig. S11, A and B). HLA-DR^{int}PD-L1^{-/lo}CD14⁺ monocytes showed less heterogeneity than their counterparts and had negligible or suppressive effects on Ly95 cell response [Fig. 7B, (blue box) and C, and fig. S11B]. These data indicate that various effects of bystander TAMs on tumor-specific T cells interacting with targets appeared not to be associated with the degree of PD-L1 expression on TAMs. In some patients with LC, PD-L1^{hi} TAMs were able to augment tumor-specific T cells and were thus functionally equivalent to M1 macrophages, despite their increased expression of CD206 and CD163 M2-like markers.

We also found that, despite the noticeably high PD-L1 surface expression on TAMs, when these HLA-A2⁺ cells were loaded with the NY-ESO peptide, they were still able to directly stimulate Ly95 cell responses. However, this response was increased in the presence of PD-L1-blocking mAbs, suggesting that TAM-derived PD-L1 was functional and could regulate the magnitude of Ly95 T cells response (fig. S11C). Importantly, in this two-component model, HLA-A2⁺/NY-ESO peptide complex-bearing TAMs did not act as bystanders and formed direct immunological synapses with cognate Ly95 cells.

Overall, our data suggest that, in the human tumor microenvironment where three major cell types (effector T cells, tumor, and myeloid cells) interact with each other, PD-L1-mediated negative regulation of tumor-specific effector T cell function takes place when the local T cells directly interact with PD-L1⁺ tumor targets. PD-L1 expressed on third-party bystander TAMs does not seem to markedly affect the effector phase of tumor-specific T cell response to tumors.

PD-L1 protects tumor peptide/MHC class I complex-bearing TAMs from being killed by cognate effector T cells

We have shown that HLA-A*02⁺ tumor-infiltrating CD14⁺ cells loaded with the NY-ESO-1 peptide could directly stimulate the NY-ESO-1-specific response of Ly95 effector T cells (Fig. 5G, and H, and fig. S11C). However, nonmalignant myeloid cells within a tumor microenvironment expressing the relevant major histocompatibility complex (MHC)/tumor antigenic peptide complexes would also present a target for elimination by preexisting cytotoxic T cells whose TCR recognizes the cognate tumor antigenic peptide. Given that

surface PD-L1 expression on TAMs is high, we hypothesized that TAM-derived PD-L1 might be involved in the protection of those TAMs expressing a relevant tumor-specific antigenic peptide from the direct killing by cognate cytotoxic CD8⁺ T cells. To test this hypothesis, in the first series of experiments, we showed that early-stage TAMs have the potential to cross-present NY-ESO-1 antigens and express the HLA-A2/NY-ESO peptide complex on the surface and thus are able to directly interact with NY-ESO-specific Ly95 T cells. Ly95 cells were mixed with flow-sorted HLA-A*0201 TAMs or T-Mos preloaded with synthetic long NY-ESO_{145–174} peptide. Similar to full-length proteins, long peptides require internalization and processing by antigen-presenting cells (APCs) for cross-presentation (31). We found that long NY-ESO_{145–174} peptide-loaded HLA-A*0201 TAMs were able to trigger IFN- γ production in CD8⁺TCRV β 13.1⁺ cells (Ly95 cells), whereas the control CD8⁺TCRV β 13.1⁻ cells in the same coculture were inactive. (Fig. 7D). These results indicate that IFN- γ production in CD8⁺TCRV β 13.1⁺ cells (Ly95 cells) was due to cross-presentation of NY-ESO-1 and not the result of allostimulation. We could not detect the IFN- γ production in Ly95 cells cocultured with T-Mos pulsed with long NY-ESO_{145–174} peptide (Fig. 7D). TAMs cross-presented NY-ESO_{145–174} peptide to a lower extent than professional APCs differentiated from HD monocytes (fig. S12A). Having analyzed several HLA-A2⁺ patients, we found that not all TAMs are able to efficiently cross-present long NY-ESO_{145–174} peptide (Fig. 7D and fig. S12B).

Next, we examined the survival of HLA-A*02⁺ monocyte-derived macrophages (Mo-Mph) that express low intensity of surface T cell cosignaling molecules (fig. S12C). These cells were pulsed with NY-ESO-1_{157–165} peptide and cocultured with Ly95 T cells for 24 hours. We found that peptide-loaded Mo-Mph were not intrinsically resistant to Ly95-mediated killing and died in the presence of the cytotoxic Ly95 T cells (Fig. 7E, top). Next, we further differentiated the Mo-Mph with TCM that resulted in the up-regulation of T cell cosignaling molecules, including PD-L1 (fig. S12C), similar to what we observed on TAMs. We found that, when TCM-differentiated Mo-Mph (TCM Mo-Mph) was preloaded with the NY-ESO-1 peptide, they had an increase in their survival after coculture with cytotoxic Ly95 cells (Fig. 7E, bottom). This acquired resistance of TCM Mo-Mph to cytotoxic Ly95 cells was partially due to their elevated expression of surface PD-L1 because Ab blockade of this receptor increased Ly95 cell-mediated lysis of these cells. Last, we performed the same experiments with freshly isolated HLA-A*02⁺ TAMs that were preexposed to NY-ESO-1_{157–165} peptide followed by cocubation with Ly95 T cells (Fig. 7F and fig. S12D). Similar to TCM-differentiated macrophages, most of the TAMs were protected from the cytolytic activity of Ly95 T cells. However, addition of PD-L1 blocking mAbs partially abrogated this protection of TAMs. These results indicate that tumor-specific cytotoxic T cells have the potential to kill bystander TAMs that express the relevant MHC class I/tumor peptide complex, but PD-L1, and possibly other T cell coinhibitory receptors, limit this process.

The presence of MMLCs, TAMs, and T-Mos is not associated with the frequency and functional heterogeneity of T cells in early-stage lung tumors

We asked whether the MMLCs and their subpopulations affect the overall survival of patients with LC. In line with other studies (14, 32), we found that the accumulation of

MMLCs, TAMs, and T-Mos in early-stage tumors does not significantly influence the overall survival of patients (fig. S13). Our correlative analyses also revealed that the difference in numbers of tumor-infiltrating MMLCs, macrophages, and monocytes were not associated with the frequency of tumor-associated neutrophils, CD8 cells, and regulatory T cells (T_{regs}) in early-stage lung tumors (fig. S14A).

Next, we determined whether the presence of $CD14^+$ MMLCs in tumors correlates with the functional status of tumor-infiltrating $CD8^+$ T cells. We found that the production of $IFN-\gamma$ by tumor-infiltrating $CD8^+$ T cells after the stimulation of tumor digests with plate-bound anti-CD3 Abs was highly heterogeneous and did not depend on the presence of MMLCs in the same tumor digests (fig. S14B). Then, we compared $IFN-\gamma$ production by tumor-infiltrating $CD8^+$ T cells with their counterparts in distant lung tissue and defined a tumor $CD8^+$ T cell response as hypofunctional if the tumor $CD8^+$ T cells produced less $IFN-\gamma$ compared to $CD8^+$ T cells in distant lung tissue. We did not find a significant correlation between the hypofunctional tumor $CD8^+$ T cells and different numbers of MMLC, macrophages, and monocytes in the same tumor digests (fig. S14C). In addition, we evaluated the production of $IFN-\gamma$ by $CD8^+$ T cells in noncancerous lung transplant tissue and determined a normal range of lung T cell responses. We then defined tumor $CD8^+$ T cells as hypofunctional if the percentage of $IFN-\gamma$ -secreting $CD8^+$ T cells was less than 1 standard deviation from the mean in the T cells in lung transplant, whereas other T cells were termed as “functional T cells”. Our correlative analysis demonstrated that tumor $CD8^+$ T cell functional status is not correlated with the accumulation of MMLCs, TAMs, and T-Mos in early-stage lung tumors (fig. S14D).

Last, we assessed whether the different abilities of tumor-infiltrating MMLCs to regulate NY-ESO-1-specific or antigen-nonspecific T cell response in vitro correlate with the accumulation and functional heterogeneity of $CD8^+$ T cells in tumors. We found that there was a strong correlation between the T cell stimulatory activity of tumor-infiltrating $CD14^+$ cells and the increased accumulation of $CD8^+$ T cells, but not of T_{regs} , in tumors (fig. S15, A and B). However, we could not find any associations between the ability of tumor-infiltrating $CD14^+$ cells to regulate antigen-specific and nonspecific T cell responses in vitro and T cell functional heterogeneity in early-stage tumors (fig. S15, C to E).

DISCUSSION

Our findings demonstrate that the MMLCs in early-stage lung tumors consist primarily of TAMs and classical tissue monocytes existing at various frequencies among patients. We did not observe a massive accumulation of MMLCs in early-stage tumors when compared to the nonmalignant distant lung of the same patient or non-cancerous control lungs as has been seen in many advanced murine tumors (33, 34). Consistent with others (26), we also did not find the expansion of $CD14^+CD15^-HLA-DR^{lo/-}$ cells referred to as Mo-MDSCs (25) in peripheral blood, distant lung, or tumor tissues of patients with early-stage LC. Our flow cytometric phenotyping of early-stage tumor digests at the cellular level further demonstrated that the traditional M1/M2 paradigm may not be applicable to human TAMs, thus confirming the previous studies performed at the single-cell transcriptome level (19).

Instead, our data show that TAMs coexpress M1 and M2 markers rather than represent a mixture of M1 and M2 subsets.

A major finding from this study was that TAMs from most of the patients had no effect on tumor-specific T cells in vitro, with only a minority of patients having TAMs that were able to suppress effector T cell responses. In contrast, tissue monocytes tended to have suppressive effects on T cell responses, although these suppressive early-stage HLA-DR^{int}CD14⁺ tissue monocytes did not exhibit the defining HLA-DR^{lo/-}CD14⁺ phenotype of Mo-MDSCs (25). The diversity of functional effects of TAMs was not associated with classic M1 or M2 macrophage marker expression. Thus, our study indicates that the definition of a macrophage's putative function based solely on classical M1/M2 expression markers can lead to misinterpretation of the true role of TAMs in regulation of effector tumor-specific T cell responses.

A key question for checkpoint inhibitor therapy is the relative contribution of PD-L1 expressed on tumor versus myeloid cells to suppression of the effector phase of T cell responses. Recently, studies using mouse models with genetic deletion of PD-L1 demonstrated that host-derived PD-L1 could mediate suppression of antitumor immunity in vivo but was highly dependent on the tumor model (17, 18, 35). We demonstrated that, in humans, only PD-L1 on target tumor cells clearly inhibited the effector functions of T cells; however, we did not observe a significant link between PD-L1 expression on third-party bystander TAMs and the suppression of effector T cells interacting with targets. Our results could be explained by recent studies, which demonstrate that the clustering of PD-1 and its colocalization with TCRs in the immune synapse upon binding to its ligands are absolutely required for subsequent suppression of T cell response (36). Our data demonstrate that TAM-derived PD-L1 exerted a regulatory role only during the interaction of TAMs presenting relevant peptide with cognate effector T cells. Our findings may add yet another piece of evidence to the ongoing discussion on why evaluation of PD-L1 alone is an imperfect diagnostic biomarker and why some patients with strong PD-L1 expression do not respond to PD-1/PD-L1 blockade therapy (1, 37). Our results suggest that measuring PD-L1 expression in both tumor MMLC and tumor cells may more faithfully predict the therapeutic benefit of PD-1/PD-L1 blockade in patients with LC.

Our data also suggest that a possible function of TAM-derived PD-L1 might be to restrain the magnitude of effector T cell activation and to “protect” the TAM from cell lysis by effector T cells. Studies in mice support the idea that, in addition to tumor targets, cytotoxic T cells are also able to kill “innocent” APCs that express the cognate MHC class I/peptide complex in vivo (38). We found that high PD-L1 surface expression prevented the TAMs that were expressing a MHC class I/peptide from being targeted by tumor-specific effector T cells. Our results thus suggest a scenario where PD-L1 blockade therapy might lead to opposite outcomes in patients with cancer. On the one hand, blockade of anti-PD-L1 on tumor cells would increase the cytotoxic activity of tumor-specific effector T cells toward these tumor cells. On the other hand, the blockade of PD-L1 on professional APCs might lead to their elimination by preexisting tumor-specific cytotoxic T cells that might result in the inability to maintain the stimulation and the longevity of existing antitumoral effector T cell responses in tumors.

The observation that human TAMs in early-stage LC are not M2-polarized macrophages or primarily T cell suppressive suggest that some TAM-directed therapies that looked promising in mouse models (39, 40) might not show the significant antitumor activity in early-stage human tumors. Our data strongly suggest that strategies to validate results from animal models for translation to human immunobiology are required to advance current approaches in immunotherapy. The knowledge of the species discrepancies in the biology of tumor-associated myeloid cells can be very useful in improving the mouse models to reflect more closely the human tumor microenvironment, especially during the early stage of tumor development.

This study has certain limitations and has raised several interesting but still unanswered questions. The major limitation is that we investigated the role of tumor MMLCs in the regulation of tumor-specific T cell response in vitro that may not faithfully reflect events in tumors of patients with cancer. We did not systematically study potentially important nonimmunologic properties of the TAMs that might support tumor progression, invasion, and neoangiogenesis. We still do not know the precise mechanisms by which CD14⁺ MMLCs, isolated from tumors of minority of early-stage patients, suppress T cell responses. The explanation does not appear to lie with the “classic” mediators of T cell inhibition. To study the ability of tumor MMLCs, we used NY-ESO-1 long peptides that might not faithfully represent the physiological conditions and the relevant source of tumor antigen requiring for cross-presentation by tumor MMLCs in vivo. Thus, the relevant source of tumor antigens required for cross-presentation by tumor MMLCs in vivo remains to be elucidated. We also do not know whether, in advanced tumor stages, a more fully developed suppressive environment will drive monocytes to differentiate into more discrete and stable protumor TAMs that resemble murine TAMs and thus might be more amenable to these TAM-targeted therapies. Gaining a more complete understanding of TAM functions in a variety of human tumors at different stages will help us to more accurately apply new therapeutic strategies or to redesign existing therapeutic strategies to regulate the function of tumor-infiltrating MMLCs.

MATERIALS AND METHODS

Study design

The objective of this study was to determine the phenotypic composition of tumor-infiltrating MMLCs and to assess how different cell populations of this lineage regulated the effector phase of tumor-specific T cell responses. Flow cytometry and immunohistochemistry were used to assess the phenotype and function of MMLCs in a single-cell suspension obtained from freshly resected human lung tumor and distant tissue. NY-ESO-1-specific Ly95 T cells and A549 human lung AC cell line expressing NY-ESO-1 protein and HLA-A*02 were used to analyze the effector phase of T cell responses (28, 41). A total of 169 random patients with stage 1 and 2 LC, who were scheduled for surgical resection, were consented for collection of a portion of their tumor tissue and/or blood for research purposes at the Hospital of the University of Pennsylvania after obtaining consent that had been approved by Institutional Review Board (protocol no. 805800). All patients selected for entry into the study met the following criteria: (i) histologically confirmed

pulmonary squamous cell carcinoma or AC, (ii) no previous chemotherapy or radiation therapy within 2 years, and (iii) no other active malignancy. Detailed characteristics of the patients can be found in table S1. Investigators conducting the reverse transcription quantitative polymerase chain reaction, enzyme-linked immunosorbent assay, and metabolite assays were blinded as to whether the samples were derived from tumor, distant lung tissue, or blood. Primary data for all experiments where $n < 20$ are reported in data file S1.

Statistics

All data were tested for normal distribution of variables using D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests. Sample distribution was considered normal if the data passed one of those tests. Comparisons between two groups were assessed with a two-tailed Student's *t* test for paired and unpaired data if data were normally distributed. Nonparametric Wilcoxon matched-pairs test and Mann-Whitney unpaired test were used when the populations were not normally distributed. Likewise, multiple groups were analyzed by one-way ANOVA with corresponding Tukey's multiple comparison test if normally distributed or by the Kruskal-Wallis (for unpaired) data and Friedman (for paired data) tests with Dunn's multiple comparison test if not normally distributed. Nonparametric Spearman or parametric Pearson test were used for correlation analysis. The relationship between survival and the frequency of MMLCs and their populations was estimated by the Kaplan-Meier method. All statistical analyses were performed with GraphPad Prism 6. A *P* value < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Frequencies of tumor-infiltrating macrophage/monocytes.

(A) Frequencies of tumor myeloid CD11b⁺ cell populations and morphology of tumor CD14⁺ cells. Scale bar, 10 μ m. SSC-A, side scatter area. (B) CD14⁺ MMLC frequencies in tumor, distant lung tissue, and blood of patients with LC, as well as in noncancerous lung transplant (LT) and HD blood. (C) The expression of macrophage-associated markers on CD11b⁺CD14⁺ cells in digested tumors and blood of patients with LC. (D) The proportion of CD14⁺ cells expressing macrophage markers. MFI, mean fluorescence intensity. (E) CD68⁺ cells in tumor islet and tumor stroma with CD68 (brown) and cytokeratin (red). Scale bar, 100 μ m. (F) HLA-DR expression in HD blood and on gated CD14⁺ cells. (G and H) The proportion of CD14 cells expressing low and high HLA-DR. (I) The morphology of tumor HLA-DR^{hi} and HLA-DR^{int} CD14⁺ cells. Scale bars, 10 μ m. Kruskal-Wallis (B and D: CD206 and CD163; G and H, left), Wilcoxon paired test (E), and Friedman tests (G and H, right), one-way analysis of variance [ANOVA; D: CD115 and interferon (IFN) regulatory factor 8 (IRF8)]. The red line represents mean $\pm$ SEM; *** P < 0.001, ** P < 0.01, and * P < 0.05. The number of patients included in each analysis is indicated on the graphs.

Fig. 2. Mixed phenotype of NSCLC-associated macrophages.

(A) Flow cytometric analysis of HLA-DR expression and macrophage markers on CD11b⁺CD14⁺ cells in tumor and peripheral blood mononuclear cells (PBMCs) of patients with LC. (B) The correlation between the proportions of CD14⁺ cells expressing HLA-DR and macrophage markers among all live CD11b⁺CD14⁺ cells in tumors. Spearman test (HLA-DR^{hi}CD206, HLA-DR^{hi}CD40, HLA-DR^{hi}CD86, and HLA-DR^{hi}CD163) and Pearson test (HLA-DR^{hi}CD80). (C) Cumulative flow cytometry results showing the frequency of cells coexpressing HLA-DR and macrophage markers among all live CD11b⁺CD14⁺ cells in tumors, distant lung tissue of patients with LC, and noncancerous lung transplant (LT). (D) Cumulative flow cytometry results showing TAM frequencies among all nucleated cells in tumors, distant lung tissue of patients with LC, and noncancerous lung transplant. TAMs were defined as CD11b⁺CD14⁺CD66b⁻HLA-DR^{hi}CD206^{hi}CD40^{hi} cells. (E) Flow cytometric analysis of the expression of macrophage markers on TAMs and M1/M2 macrophages differentiated in vitro. Representative histograms from one of five experiments

are shown. **(F)** Heat map of the proportions of tumor CD14⁺ cells expressing macrophage markers (rows) across studied patients (columns). Kruskal-Wallis with Dunn's multiple comparisons test [C (HLA-DR^{hi}CD206) and D], one-way ANOVA with Turkey's multiple comparisons tests (C: HLA-DR^{hi}CD40, HLA-DR^{hi}CD80, HLA-DR^{hi}CD86). The red line represents mean $\pm$ SEM; *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. The number of patients included in each analysis is indicated on the graphs.

Fig. 3. Expression of T cell cosignaling receptors on MMLCs.

(A) Cumulative results showing the proportion of CD14⁺ cells expressing T cell cosignaling molecules in tumors, distant lung tissue, peripheral blood of patients with LC, and noncancerous lung transplant (LT). (B) Expression of PD-L1 (brown) in lung tumor tissue. Scale bar, 100 μ m. (C) Expression of PD-L1 and CD14 in digested tumor, distant tissue, and PMBCs. (D) Cumulative results showing the intensity of PD-L1 expression on CD14⁺ and EpCam⁺ cells in tumors. (E) Expression of HLA-DR and PD-L1 on live-gated CD11b⁺CD14⁺CD66b⁻ cells in tumor and PBMCs. (F) Correlation between the proportions of CD14⁺ cells expressing HLA-DR and PD-L1 among all live CD11b⁺CD14⁺ cells in tumors. (G) Summary results showing the proportion of HLA-DR^{hi}PD-L1^{hi} cells among all CD11b⁺CD14⁺CD66b⁻ cells in tumor, distant lung tissue of patients with LC, and noncancerous lung transplant. (H) Representative dot plots demonstrating the expression of PD-L1 and indicated T cell costimulatory receptors on live-gated CD11b⁺CD14⁺ cells in tumor and PBMC. (I) Heat map of the proportions of CD14⁺ cells expressing T cell cosignaling receptors across studied patients. One-way ANOVA (A: CD40^{hi}CD14, CD54^{hi}CD14, 4-1BBL^{hi}CD14, PD-L1^{hi}CD14, B7-H3^{hi}CD14, and PD-L2^{hi}CD14), Kruskal-Wallis test [A (CD86^{hi}CD14) and G], paired *t* test (D), and Spearman test (F) were performed. The red line represents mean $\pm$ SEM; ****P* < 0.001, ***P* < 0.01, and **P* < 0.05. The number of patients included in each analysis is indicated on the graphs.

Fig. 4. Effects of tumor CD14 cells on antigen-nonspecific T cell responses.

(A) Neutral (blue box), stimulatory (green box), and suppressive (red box) effects of tumor CD14⁺ cells from different patients on the IFN- γ production by autologous CD8⁺ T cells stimulated with plate-bound anti-CD3 Abs. (B) Summary results of IFN- γ production by stimulated autologous CD8⁺ T cells in the presence of CD14⁺ cells isolated from tumor, distant tissue, and blood. Stimulatory index (SI) is a ratio (CD8 cells + CD14 cells)/(CD8 cells). (C) Proliferation of stimulated autologous CD8⁺ T cells in the presence of CD14⁺ cells from tumor, distant tissue, and blood. CFSE, carboxyfluorescein succinimidyl ester; BrdU, bromodeoxyuridine. (D) Cumulative results of autologous CD8⁺ T cell proliferation stimulated with anti-CD3 Abs in the presence of CD14⁺ cells from tumor, distant tissue, and blood. SI is a ratio (CD8 + CD14)/(CD8). (E and F) Representative dot plots and cumulative results showing the effect of CD14⁺ cells on IFN- γ production by CD8⁺ T cells in digested tumor, distant tissue, and PBMCs that were depleted for CD14⁺ cells before stimulation with plate-bound anti-CD3 Abs. SI is a ratio (IFN- γ ⁺CD8⁺ cells in total digest)/(IFN- γ ⁺CD8⁺

cells in CD14 cell depleted digest). One-way ANOVA (B) and Friedman (D and F) tests were performed for two groups, where SI is >1 and <1 , $*P < 0.05$. The number of patients included in each analysis is indicated on the graphs.

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Fig. 5. Effects of tumor CD14 cells on the effector phase of NY-ESO-1-specific T cell responses. (A) Activation and IFN- γ production by Ly95 cells stimulated with A549 or A549/A2-NY-ESO-1 tumor cells. (B) Suppressive (red box), neutral (blue box), and stimulatory (green box) effects of tumor CD14 cells from different patients on Ly95 T cell response. (C) Cumulative results showing the different effects of tumor CD14⁺ cells on Ly95 cell response. SI is a ratio (Ly95 + A549-NY-ESO-1 + CD14)/(Ly95 + A549-NY-ESO-1). One-way ANOVA test for two groups, where SI is >1 and <1, * P < 0.05. (D) IFN- γ production by Ly95 cells that were either simultaneously mixed with tumor CD14 cells and A549/A2-NY-ESO-1 cells (Tumor CD14 day 0) or preincubated with CD14 cells for 24 hours before stimulation with A549/A2-NY-ESO-1 cells (Tumor CD14 day 1). Representative results from one of five experiments are shown. (E and F) Effect of CD14 cells on the tumoricidal activity of Ly95 cells toward A549/A2-NY-ESO-1 tumor cells. Tumoricidal index is a ratio (Ly95 + A2-NY-ESO-1 + CD14)/(Ly95 + A2-NY-ESO-1). Friedman test for two groups, where SI is >1 and <1, * P < 0.05. (G and H) IFN- γ production by Ly95 T cells stimulated with A549/A2-NY-ESO-1 cells or tumor HLA-A2⁺CD14 cells pulsed with NY-ESO-1 peptide (Tumor CD14). One-way ANOVA, * P < 0.05 and *** P < 0.001. The number of patients included in each analysis is indicated on the graphs.

Fig. 6. Effects of TAMs and tumor-expressed PD-L1 on the effector phase of tumor-specific T cell responses.

(A) Representative dot plots demonstrating the NY-ESO-1-specific Ly95 cell response (IFN- γ production and killing activity) to control A549 and PD-L1^{hi} A549/A2-NY-ESO-1 tumor cells. (B) Cumulative results showing the NY-ESO-1-specific Ly95 cell IFN- γ production to A549/A2-NY-ESO-1 and A549/A2-NY-ESO-1 PD-L1^{hi} tumor cells in the presence or absence of PD-L1-blocking Abs. Repeated measures (RM) one-way ANOVA with Turkey's multiple comparisons tests were performed for two groups, where SI is >1 and <1, *** P < 0.001. (C) The effects of tumor PDL-1^{hi} and PD-L1^{lo} CD14 cells isolated from different patients on the IFN- γ and granzyme B production by Ly95 cells cocultured with A549/A2-NY-ESO-1 tumor cells in the presence or absence of PD-L1-blocking Abs. (D) The proportions of HLA-DR^{hi}PD-L1^{hi} cells among tumor CD14⁺ cells that had an SI of more than 1 (SI > 1) and less 1 (SI < 1) (mean $\pm$ SEM). Unpaired t test, ** P < 0.01. (E) Heat map demonstrating the relationship of the SIs of tumor CD14⁺ cells and their relative proportions expressing T cell costimulatory and coinhibitory receptors (rows) across the studied patients (columns). The number of patients included in each analysis is indicated on the graphs.

Fig. 7. Role of TAM-derived PD-L1 in the regulation of tumor-specific T cell responses. (A) The gating strategy for sorting CD14⁺HLA-DR^{hi}PD-L1^{hi} TAMs and CD14⁺HLA-DR^{int}PD-L1⁻ T-Mos from tumors. (B and C) Representative dot plots and cumulative results showing the IFN- γ and granzyme B production by Ly95 cells cocultured with A549/A2–NY-ESO-1 tumor cells in the presence or absence of CD14⁺HLA-DR^{hi}PD-L1^{hi} and CD14⁺HLA-DR^{int}PD-L1⁻ cells sorted from tumors. SI is a ratio (Ly95 + A549-NY-ESO-1 + CD14)/(Ly95 + A549-NY-ESO-1), paired *t* test, ***P* < 0.01. (D) TAMs and T-Mos were preincubated with long NY-ESO_{145–174} peptide at a concentration of 10 μ g/ml for 2 hours before mixing with Ly95 cells. NY-ESO-1–specific IFN- γ production was measured by flow cytometry in live-gated CD8⁺TCRV β 13.1⁺ and CD8⁺TCRV β 13.1⁻ cells. Mann-Whitney test, **P* < 0.05. (E) Representative dot plots and cumulative results demonstrating the cytotoxic activity of Ly95 T cells toward NY-ESO-1 peptide–pulsed HLA-A2⁺CD14 macrophages differentiated from blood monocytes in the presence of M-CSF (Mo-Mph) or M-CSF and tumor-conditioned media (TCM; TCM Mo-Mph) in vitro. (F) Representative dot plots and cumulative results demonstrating the cytotoxic activity of Ly95 T cells toward NY-ESO-1 peptide–pulsed HLA-A2⁺CD14 cells isolated from tumors. Wilcoxon paired test, **P* < 0.05. The number of patients included in each analysis is indicated on the graphs.