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Presence of innate lymphoid cells in pleural effusions of primary and metastatic tumors: Functional analysis and expression of PD-1 receptor

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The tumor microenvironment (TM) contains a wide variety of cell types and soluble factors capable of suppressing immune responses. While the presence of NK cells in pleural effusions (PE) has been documented, no information exists on the presence of other innate lymphoid cell (ILC) subsets and on the expression of programmed cell death-1 (PD-1) in NK and ILC. The presence of ILC was assessed in PE of 54 patients (n = 33 with mesothelioma, n = 15 with adenocarcinoma and n = 6 with inflammatory pleural diseases) by cell staining with suitable antibody combinations and cytofluorimetric analysis. The cytokine production of ILC isolated from both PE and autologous peripheral blood was analyzed upon cell stimulation and intracytoplasmic staining. We show that, in addition to NK cells, also ILC1, ILC2 and ILC3 are present in malignant PE and that the prevalent subset is ILC3. PE-ILC subsets produced their typical sets of cytokines upon activation. In addition, we analyzed the PD-1 expression on NK/ILC by multiparametric flow-cytometric analysis, while the expression of PD-1 ligand (PD-L1) was evaluated by immunohistochemical analysis. Both NK cells and ILC3 expressed functional PD-1, moreover, both tumor samples and malignant PE-derived tumor cell lines were PD-L1$^+$ suggesting that the interaction between PD-1$^+$ILC and PD-L1$^+$tumor cells may hamper antitumor immune responses mediated by NK and ILC.

Introduction

The tumor microenvironment (TM) contains a wide variety of malignant cells and an assortment of different nonmalignant cell types including mesenchymal stromal cells,[1] endothelial cells,[2] lymphoid cells,[1] macrophages and other myeloid cells[3,4] as well as soluble factors and cytokines released from the tumor cells themselves or tumor-associated (TA) cells.[5,6]

Five major groups of ILCs have been defined based on the cytokine produced and the transcription factors required for their development. They include natural killer (NK) cells, group 1 ILC (ILC1), group 2 ILC (ILC2), group 3 ILC (ILC3) and lymphoid tissue-inducer (LTi) cells. ILC are predominantly tissues-resident cells that, different from T and B lymphocytes, lack antigen-specific receptors.[7] Notably, ILC1, ILC2 and ILC3

Key words: malignant pleural effusions, innate lymphoid cells, adenocarcinoma, mesothelioma, PD-1, PD-L1, checkpoint inhibitors

Abbreviations: FcγR: Receptors for the constant fragment of immunoglobulin gamma chain; HD: healthy donors; ILC: Innate lymphoid cell subsets; mPE: malignant pleural effusions; NK: Natural killer; PB: Peripheral blood; PD-1: Programmed cell death-1; PD-L1: PD-1 ligand; TF: Transcription factor; Th: T helper; TLS: tertiary lymphoid structures; TM: tumor microenvironment

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What's new?

Pleural tumors result in effusions that are not well characterized. In this study, the authors found that pleural effusions from patients with primary mesothelioma or metastatic adenocarcinoma contain NK cells and other innate lymphoid cells (ILC). These immune cells were capable of expressing normal cytokines, including the checkpoint protein PD-1. However, the tumor cells were found to express the ligand PD-L1. These results suggest a PD-1-mediated inhibitory effect on lymphoid cells with potential anti-tumor activity. Better understanding of this inhibition in the tumor microenvironment may lead to new targets for checkpoint-inhibitor therapies.

mirror corresponding T helper cell subsets in terms of cytokine production (T helper (Th) 1, Th2 and Th17 cells, respectively) and are involved in defenses against pathogens, inflammatory responses and also in tissues remodeling and lymphoid organogenesis.[8,9] In particular, ILC1, similarly to NK cells, produce IFN-γ while ILC2 mainly release type-2 cytokines, such as IL-5, IL-13 and small amounts of IL-4. Finally, ILC3, further subdivided into NCR^+ and NCR^-ILC3, prevalently secrete IL-22 and IL-17, respectively.[10,11]

NK cells, representing cytotoxic ILC, constitutively express perforin and granzymes, rapidly kill tumor or virus-infected cells (particularly when lacking MHC-I expression) and release cytokines.[12] While the relevance of NK cells in the control of tumor growth and metastasis has been well documented, limited information exists in humans on whether other ILCs play a role in tumor immunity.[13]

Tumor cells frequently display an altered expression of MHC-I on their surface and acquire ligands recognized by activating NK receptors, thus becoming potentially susceptible to NK cell-mediated killing. On the other hand, the TM may inhibit the expression of activating NK receptors and compromise the effector function of NK cells. Another mechanism by which tumors can escape the control of both NK and T lymphocytes is to induce in these cells the *de novo* expression of inhibitory receptors as well as their ligands on tumor cells. This allows tumor cells to avoid killing and to establish an immunosuppressive microenvironment.[14–24]

Regarding ILC, their ability to regulate/promote inflammatory processes, to mediate neoangiogenesis and form tertiary lymphoid structures (TLS) suggests that they may exert either a pro-tumor or an antitumor effect depending on the tumor type and on the cellular and soluble components of TM.[25] Thus, ILCs may sustain tumor growth by secreting cytokines that favor an immunosuppressive TM leading to tumor immune-escape. On the other hand, they may favor immune responses through the recruitment of effector cells at the tumor site.[26–28] In this context, ILC3 have been shown to support the formation of TA-TLS that favor the capture and the presentation of tumor antigens to T lymphocytes and the initiation of tumor-specific immune responses.[29] In a previous study, we showed that NK cells present in malignant pleural effusions (mPE) are not anergic, as they can release cytokines, and kill efficiently tumor targets including autologous tumor cells.[30–32] However, no information is available on the actual presence and on the possible effect of other ILC subsets in mPE derived from patients with primary or metastatic tumors.

The programmed death-1 (PD-1, CD279) receptor is an important checkpoint involved in peripheral immune tolerance, thanks to its ability to inhibit cytolytic effector T cells, to prevent their attack towards normal tissues and to control the overreaction of the immune system and consequent tissue damages.[33–37] PD-1 pathway may sharply inhibit the function of effector cells, potentially able to kill tumor cells, including cytolytic T lymphocytes and NK cells, through the interaction with their corresponding ligands (PD-L1/2) expressed on tumor cells.[38–45] Recent studies, in patients with ovarian carcinoma, have shown that NK cells may express PD-1. Notably, these $PD\text{-}1^+$ cells were much more abundant in ascitic fluid than in peripheral blood of the same patient.[46]

In the present study, we show that PE from primary (mesothelioma) or metastatic (adenocarcinoma and carcinoma) tumors, in addition to NK cells, contain ILCs. ILC3 represent the prevalent PE-ILC subset. Upon activation, all ILC isolated from mPE released their typical cytokines. Further analysis revealed that both NK cells and ILC3 express functional PD-1 suggesting that its expression may cause an impairment of their antitumor activity.

Materials and Methods

Patients and cells

We collected 54 pleural effusions (PE) obtained from thoracentesis in patients with primary or metastatic tumor of different origin and with inflammatory disorders as described in Table 1 and in Table S1. PE cells were obtained by centrifugation at 400*g* for 10 min and preserved in 10% serum-supplemented RPMI 1640 medium (BioWhittaker, Lonza). This study was approved by Azienda Sanitaria Locale 3 (ASL, Genova, Italy) Ethics Board (ID 33533184, 29/10/2013). Peripheral blood (PB) of healthy donors (HD) from buffy coat (UO Centro Trasfusionale, IRCCS AOU San Martino-IST) was used as controls. All patients gave consent according to the Declaration of Helsinki. Lymphocytes from PE and PB were obtained by density gradient separation Ficoll–Hypaque (Lympholyte-H, Cederlane) as previously described[30] and subsequently used for phenotypic and functional analysis.

Flow cytometry analyses and monoclonal antibodies

Cells were stained with the following mAbs: IL-17A-FITC; IL-17A-APC; IFN-γ-PE CD335 (NKp46)-APC; CD294 (CRTH2)-PE; CD117-APC; PD-1-APC, PD-1-PE, CD25-PE, CD123-FITC and CD3-PE, purchased from Miltenyi; NKp46-V450, IFN-γ-PE and CD45-APC-H7 purchased from BD; IL-22-PE; TNF-α-eFluor450; IL-13-PE, IL-4-PE, IL-5-PE, RORγt-PE; and RORγt-APC; purchased from eBioscence; CD94-FITC; CD127-BrilliantViolet421;

Table 1. Features of patients included in the study

Patients with metastatic tumors	
n = 15, median age 71.6 (range 55–91):	n = 7 Lung adenocarcinoma
Male: n = 8 median age 70.1 (range 55–91)	n = 3 Intestinal adenocarcinoma
Female: n = 7 median age 73.2 (range 64–83)	n = 2 Uterine carcinoma
	n = 1 Breast carcinoma
	n = 1 Pancreatic adenocarcinoma
	n = 1 Bladder carcinoma
Patients with primary tumors	
n = 33, median age 76.9 (range 53–89):	n = 12 Epithelioid mesothelioma
Male: n = 28 median age 77.8 (range 55–89)	n = 21 Mesothelioma
Female: n = 5 median age 71.6 (range 53–83)	
Patients with Inflammatory Pleural Effusion	
n = 6, median age 64.3 (range 27–76):	
Male: n = 2 median age 51 (range 27–75)	
Female: n = 4 median age 71 (range 62–76)	

NKp44-APC; NKp46-PacifiBlue; CD161-PerCP-Cy5.5; CD117-PerCP-Cy5.5; CD117-BrilliantViolet605, CD127-PerCP-Cy5.5 and CD294 (CRTH2)-APC purchased from BioLegend: IL-8-PE; NKp46-PE purchased from R&D; CD56-PC7, CD278 (ICOS)-APC, CD34-FITC, CD14-ECD, CD19-ECD and CD3-ECD purchased from Beckman Coulter; and Live/dead fixable aqua dead purchased from Invitrogen/Molecular Probes. Granzyme B-PE and perforin-FITC purchased from BD and Ancell, respectively.

For intranuclear staining of transcription factors (TF), cells were stained for surface markers, fixed and permeabilized with fixation/permeabilization buffer (eBioscience) and permeabilization buffer (eBioscience), respectively, according to the manufacturer's instructions. For intracellular cytokine detection, total lymphocytes from PE or PB were stimulated and after 18 hr, cells were stained for surface markers, fixed and permeabilized with cytofix/cytoperm (BD Biosciences, San Jose, CA) and with perm/wash (BD), respectively, according to the manufacturer's instructions. All samples were analyzed on Gallios and CytoFlex Flow Cytometers (Beckman Coulter, Brea, CA). Data analysis was done using FlowJo software (TreeStar Inc., Ashland, OR) and CytExpert (Beckman Coulter).

Cytokine production

In order to evaluate the expression of cytokines typical of different ILC subsets, we performed intracellular cytokine staining using lymphocytes obtained from either PE or PB. Total PE- or PB-lymphocytes were stimulated overnight in the presence of GolgiStop and GolgiPlug (BD), with different mixtures of cytokines as previously described:[47–50] for ILC2: IL-25 (25 ng/ml, R&D, Minneapolis, MN), IL-33 (25 ng/ml, R&D); for ILC3: IL-23, IL-1β and IL-7 (50 ng/ml each, Miltenyi Biotech, Auburn, CA); for NK cells and ILC1: Phorbol 12-myristate 13-acetate (PMA, 25 ng/ml, Sigma, St. Louis, MO) and Ionomycin (Iono, Sigma, 50 ng/ml).

In order to evaluate the functional activity of PD-1, lymphocytes derived from PE were incubated with P815 cell line (a $FcγR^+$ mastocytoma murine cell line) in the presence of anti-NKp46 (BAB281 clone, IgG1), anti-NKp30 (AZ20 clone, IgG1) and anti-NKp44 (Z231 clone, IgG1) mAbs in combination or not with anti-PD-1 mAb (PD1.3.1.3 clone, IgG2b). The E/T ratio was 1/1.

To evaluate the effect of the natural ligand of PD-1, mPE lymphocytes were incubated overnight with recombinant (r) PD-L1 protein (10 μg/ml, R&D) previously coated to plates, in the presence of PMA/Iono GolgiStop and GolgiPlug.

After stimulation, cells were stained for surface markers, fixed and permeabilized with cytofix/cytoperm (BD) and with perm/wash (BD), respectively, according to the manufacturer's Instructions.

The following mAbs were isolated in our laboratory, licensed to the indicated companies and validated for their specificity: anti-NKp30 (AZ20, IgG1), anti-NKp44 (Z231, IgG1), anti-NKp46 (BAB281, IgG1) (Beckman Coulter/Immunotech, Marseille, France). The purified anti-PD-1 mAb (PD1.3.1.3 clone, IgG2b), was originally isolated at the Laboratoire Immunologie des Tumeurs, CRCM, Marseille-Luminy (France).[40]

Immunocytochemistry

Cytology samples of pleural effusions from adenocarcinomas and mesotheliomas were immersed in acetone (3 min) and xylene (10 min) to remove the coverslip and were then rehydrated with alcohol with decreasing concentration and immersed in distilled water. The slides were stained with PD-L1 (clone SP263, Ventana Medical Systems, Tucson, AZ) on an automated staining platform (Benchmark ULTRA; Ventana) inclusive of antigen retrieval with CC1 solution (24 min) and incubation time with primary antibody (1 hr). An OptiView DAB IHC detection kit (Ventana) and an OptiView amplification kit (Ventana) were used according to the manufacturer's recommendations for the visualization of the primary anti PD-L1 antibody. The analysis was performed on 40 samples (10 derived from mesothelioma and 30 from adenocarcinoma samples). All immunocytochemistry evaluations were blindly evaluated and by two independent experts.

Results

Identification of $CD127^+$ ILC subsets in human malignant pleural effusions

We first assessed whether ILC are detectable in mPE from patients with primary (mesothelioma) or metastatic (e.g., lung and gastrointestinal adenocarcinomas and uterine carcinoma) tumors (Table 1). As shown in Figure 1, multiparametric flow cytometric analysis allows the identification of various ILC subsets. Thus, in addition to NK cells, characterized by $CD45^+CD127^-Lin^-CD56^+$ phenotype, a fraction of cells expressed the $CD45^+CD127^+Lin^-$

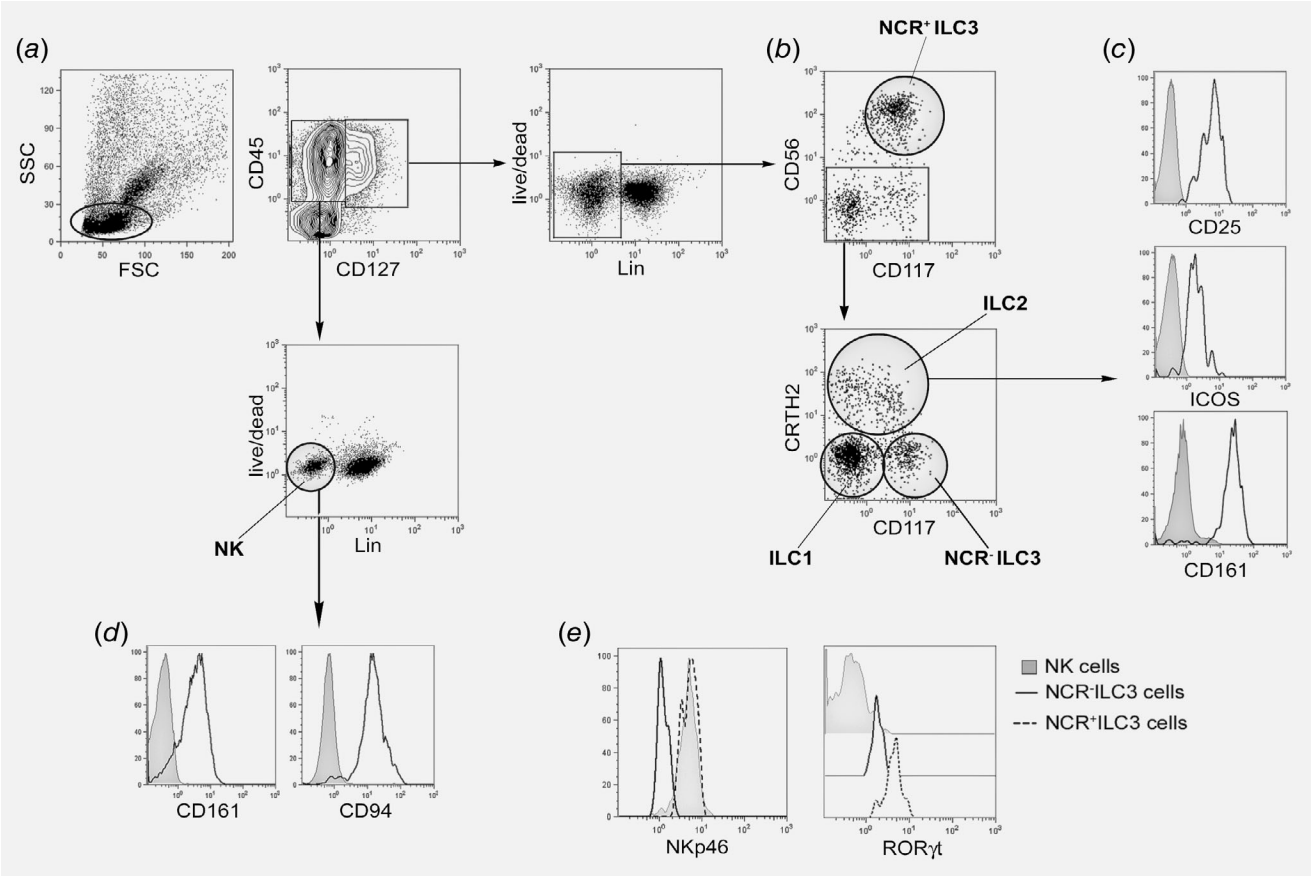

Figure 1. Identification of ILCs in mPE. (*a–e*) Flow cytometric analysis of ILC subsets present in pleural effusions of patients with primary or metastatic tumors localized in the pleural cavity. One representative experiment out of 36 performed (n = 19 mesotheliomas, and n = 11 adenocarcinomas and six control patients with inflammatory pleural diseases). Analysis of specific markers (black lines) in gated ILC2 (*c*) and NK cells (*d*), isotype control (gray profiles). (*e*) Expression of NKp46 and RORγt in NK cells (filled gray profile), NCR$^-$ILC3 (black line), and NCR$^+$ILC3 cells (dashed black line).

phenotype (Fig. 1*a*). This cell fraction contains all the ILC1, ILC2 and ILC3 subsets. Further dissection, in the CD45$^+$ population, allows to identify Lin$^-$CD127$^+$CD56$^+$CD117$^+$ cells (corresponding to NCR$^+$ILC3), while, within the CD56$^-$CD117$^{+/-}$ cell fraction, CRTH2$^+$ cells correspond to ILC2, while CRTH2$^-$CD117$^+$ and CRTH2$^-$CD117$^-$ to NCR$^-$ILC3 and ILC1, respectively (Fig. 1*b*). To further confirm such subset assignment, markers typical of different helper ILC subsets were analyzed. Thus, ILC2 expressed CD25, ICOS and CD161 (Fig. 1*c*) while NK cells were CD161$^+$ and CD94$^+$ (Fig. 1*c*). The expression of NKp46 was confined to NK cells and to NCR$^+$ILC3 (Fig. 1*e*, left panel). Both NCR$^+$ILC3 and NCR$^-$ILC3 (but not NK cells), expressed the RORγt TF, a specific marker of this ILC subset (Fig. 1*e*, right panel).

Malignant PE contains all the ILC subsets

NK cells and other ILCs present in mPE were analyzed in comparison to nonmalignant PE obtained from patients with inflammatory pleural diseases (iPE). As shown in Figure 2*a*, the CD45$^+$Lin$^-$ cell populations present in mPE and iPE contained similar proportions of NK cells and helper ILCs (ILC1/2/3).

Since, besides NK cells, also other ILC subsets may be involved in innate responses against tumors, we assessed the relative proportions of helper ILC subsets. The analysis of CD45$^+$Lin$^-$CD127$^+$ cells including all helper ILC (and not NK cells) revealed that the relative proportions of ILC1/ILC2/ILC3 were similar in mPE and iPE (Fig. 2*b*). In particular, the proportions of ILC1, ILC2 and ILC3 in mPE were 14, 14 and 72%, respectively, and 5% ILC1, 9% ILC2 and 86% ILC3 in iPE (Fig. 2*b*).

We then asked whether mPE derived from mesotheliomas (primary) or adenocarcinomas (metastatic) tumors may differ in terms of ILCs composition. As shown in Figure 3*a*, no substantial differences existed between the two tumor histotypes. Further dissection confirmed that ILC3 were prevalent in both tumor histotypes (Fig. 3*b*), while ILC1 and ILC2 were 12.1 and 16.9% in mesothelioma and 15.6% and 11.6% in adenocarcinoma, respectively. The assessment of the two main ILC3 subsets, that is, NCR$^+$ and NCR$^-$ ILC3 revealed that both subsets were present in the mPE of both mesotheliomas and adenocarcinomas (Fig. 3*c*).

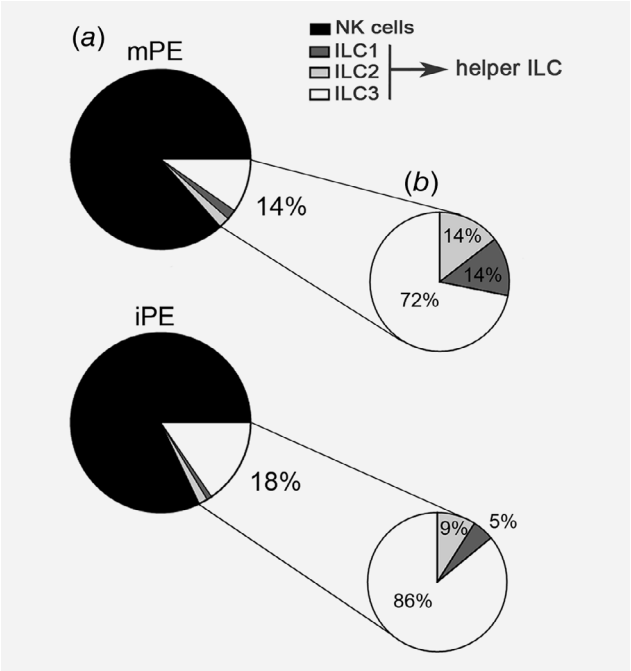

Figure 2. Presence of different proportions of ILCs in mPE and iPE. (*a*) Proportions of NK cells and helper ILCs in the CD45$^+$Lin$^-$ cell fraction detected either in mPE (n = 30) or in iPE (n = 6). (*b*) Relative proportions of ILC1 (dark gray), ILC2 (light gray) and ILC3 (white) in CD45$^+$Lin$^-$CD127$^+$ cells in malignant or inflammatory PE.

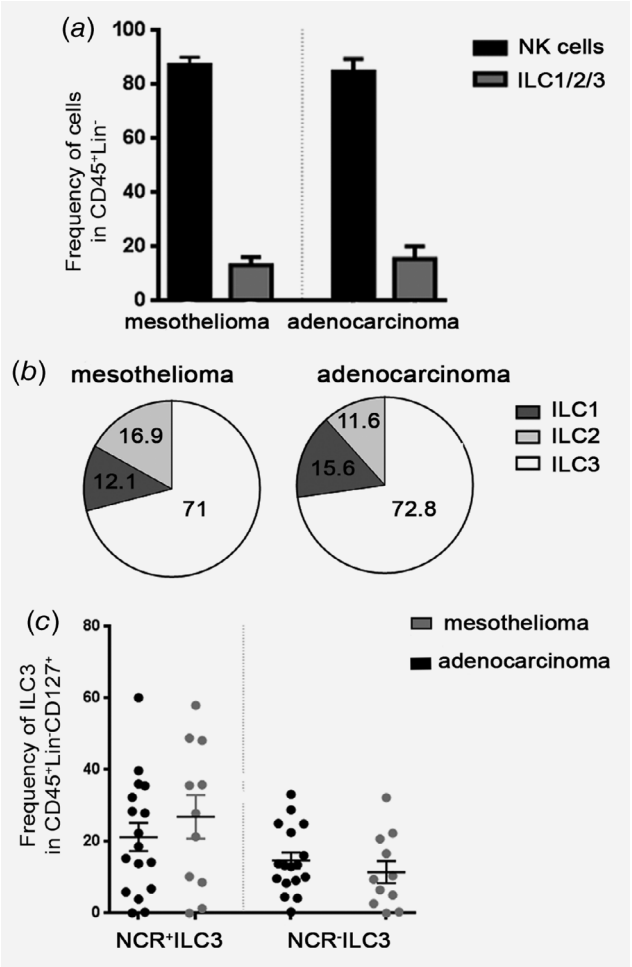

Figure 3. PE-derived ILC3 are the predominant subset in primary and metastatic tumors. (*a*) Percentages of NK cells (black bars) and ILC1/2/3 (gray bars) in the CD45$^+$Lin$^-$ cell fraction present in mesothelioma and adenocarcinoma derived PE. (*b*) Proportion of ILC1 (dark gray), ILC2 (light gray) and ILC3 (white) gated on CD45$^+$Lin$^-$CD127$^+$ cells in mesothelioma and adenocarcinoma PE. (*c*) Percentage of NCR$^+$ILC3 and NCR$^-$ILC3 gated on CD45$^+$Lin$^-$CD127$^+$ cells in mesothelioma (gray dots) and adenocarcinoma (black dots) PE.

Cytokine production by ILC present in mPE

Different ILC subsets secrete peculiar sets of cytokines which are the main mediators of their functional activities. In order to assess the pattern of cytokines produced by mPE ILCs, lymphocytes isolated from mPE were stimulated and analyzed by flow cytometry. As shown in Figure 4*a* and 4*b*, IFN-γ and TNF-α were mainly produced by NK cells and ILC1, while IL-22 and IL-8 by NCR$^+$ILC3 and IL-17 by NCR$^-$ILC3. Moreover, the analysis of cytolytic granule content revealed that mPE-NK cells and -ILC3 expressed granzyme B and perforin at lower level than PB-NK cells (Supporting Information Fig. S1).

Since ILC2 are detectable also in peripheral blood (PB), we analyzed mPE ILC2 in comparison with those present in autologous PB. Similar percentages of ILC2 producing IL-13 and IL-5 were detected in mPE and autologous PB. Remarkably, PE-ILC2 produced much higher IL-4 as compared to autologous PB-ILC2 (Fig. 4*c*). Taken together, these data indicate that human mPE contain functional ILC, capable of secreting cytokines typical of given ILC subsets.

mPE-ILC express high levels of PD-1 inhibitory checkpoint

Inhibitory checkpoints may play a critical role in the inhibition of anti-tumor effector responses. Thus, we investigated whether NK cells and other ILC subsets present in mPE would express PD-1. Multiparametric flow-cytometric analysis revealed that both NK cells and ILC3 isolated from mPE express PD-1 in variable proportions, while NK cells derived from autologous PB and of healthy donors (HD) PB expressed very low levels of PD-1 (Fig. 5*a*). Notably, both PD-1$^+$NK cells and PD-1$^+$ ILC3 were enriched in mPE. While CD56^{bright} outnumbered the CD56^{dim} NK cells in mPE,[30] the PD-1 expression was confined to the CD56^{dim} subset (not shown). These data are in agreement with previous findings on NK cells present is ascitic fluid of patients with ovarian carcinoma.[46]

To further study whether the PD-1 inhibitory pathway may be induced in NK cells and ILC3 associated with pleural tumors, we investigated whether PD-L1, the major PD-1 ligand, is expressed by tumor cells. As shown in Figure 5*b* and 5*c*, in tumor specimens of adenocarcinoma cells expressed PD-L1. Moreover, PD-L1 was expressed by all *in vitro* stabilized cell lines derived from mPE of patients with mesothelioma or adenocarcinoma (data not shown). We next investigated whether

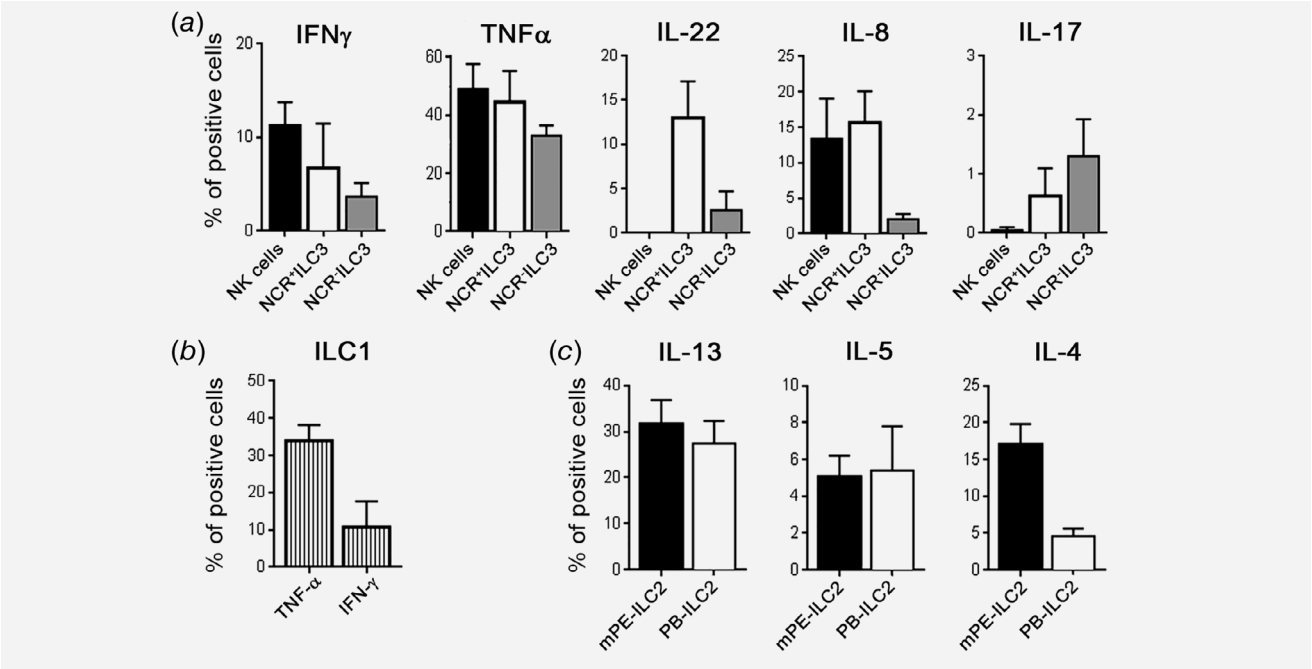

Figure 4. ILC1, ILC2 and ILC3 present in mPE produce their typical cytokines upon activation. (*a–c*) Freshly isolated mPE-lymphocytes were stimulated (see Materials and Methods) for 18 hr and analyzed for IFN-γ, TNF-α, IL-22, IL-8, IL-17, IL-13, IL-5 and IL-4 expression by intracytoplasmic staining. (*a*) Production of informative specific cytokines by gated NK cells (black bars), NCR^+ILC3 (white bars) and NCR^-ILC3 (gray bars; $n = 4$). (*b*) Production of IFN-γ, TNF-α by ILC1 ($n = 4$). (*c*) Specific cytokines expression by gated ILC2 present in mPE (black bars, $n = 8$) and/or corresponding autologous PB (white bars, $n = 4$).

PD-1 may inhibit the functional activity of mPE-derived NK cells and ILC3. To this end, we first investigated whether we could exploit, as a readout system, the ability of these cells to release cytokines. As shown in Figure 5*d*, mAb-mediated cross-linking of the activating natural cytotoxic receptors (NCRs) including NKp46, NKp44 and NKp30, induced cytokine production by both NK cells and NCR^+ILC3. In the presence of anti-PD-1 mAb, the NCR-mediated cytokine production was significantly inhibited (Fig. 5*d*). To further prove unequivocally the inhibitory role of PD-1 induced by its natural ligand PD-L1 (expressed by tumor cells), we performed experiments using recombinant (r) PD-L1 molecule (coated to plates). As shown in Supporting Information Figure S2, the production of TNF-α and IFN-γ by NK cells upon PD-1/PD-L1 interaction was inhibited. These data support the notion that induction of the PD-1 pathway may affect the anti-tumor activity of mPE-NK cells and -ILC3, thus favoring mechanisms of immune escape.

Discussion

In the present study, we provide the first evidence that different ILC subsets are present in the pleural fluid of primary and metastatic tumors. Thus, in addition to NK cells, ILC1, ILC2 as well as NCR^+ and NCR^-ILC3, are present in mPE and are capable of producing their typical sets of cytokines. Importantly, mPE-NK cells and -ILC3 may express functional PD-1, while mesothelioma and adenocarcinoma tumor cells express the PD-1 ligand (PD-L1). This suggests that PD-1/PD-L1 molecular interactions, at the tumor site, may compromise the antitumor activity of NK cells and ILC3.

Different ILC populations are present in tissues where they exert innate defenses against various pathogens and also contribute to tissue homeostasis and lymphoid tissue generation.[7,51] During the early phases of infections or tissue damage, ILC represent an important source of cytokines that play a relevant role in the induction of immune responses, in angiogenesis and in favoring the barrier integrity.[28,52] Recently, NK cells have been found in mPE of primary and metastatic pleural tumors. Notably, mPE-NK cells could kill tumor cells even more efficiently than autologous peripheral blood NK cells, upon short time culture in IL-15 or IL-2.[30–32] On the other hand, the possible effect of helper ILC1/2/3, on either tumor control or progression is poorly known. It is possible that through their ability to promote neoangiogenesis and secretion of type 2 cytokines (ILC2), and tissue regeneration, certain ILC subsets may favor tumor growth.[28] On the other hand, ILC3 may exert an antitumor activity thanks to the induction of tumor-associated tertiary lymphoid structures in which T cell-mediated antitumor immune responses may take place. In addition, on the basis of their known functional capabilities, they may also contribute to shaping the TM. In the present study, in an attempt to clarify whether ILC are present in PE and to assess their possible role in tumor growth, we analyzed mPE derived from primary (mesothelioma) or metastatic (adenocarcinoma) tumors. Our data provide the first evidence that different subsets of ILC are present in PE. In particular, multiparametric flow-

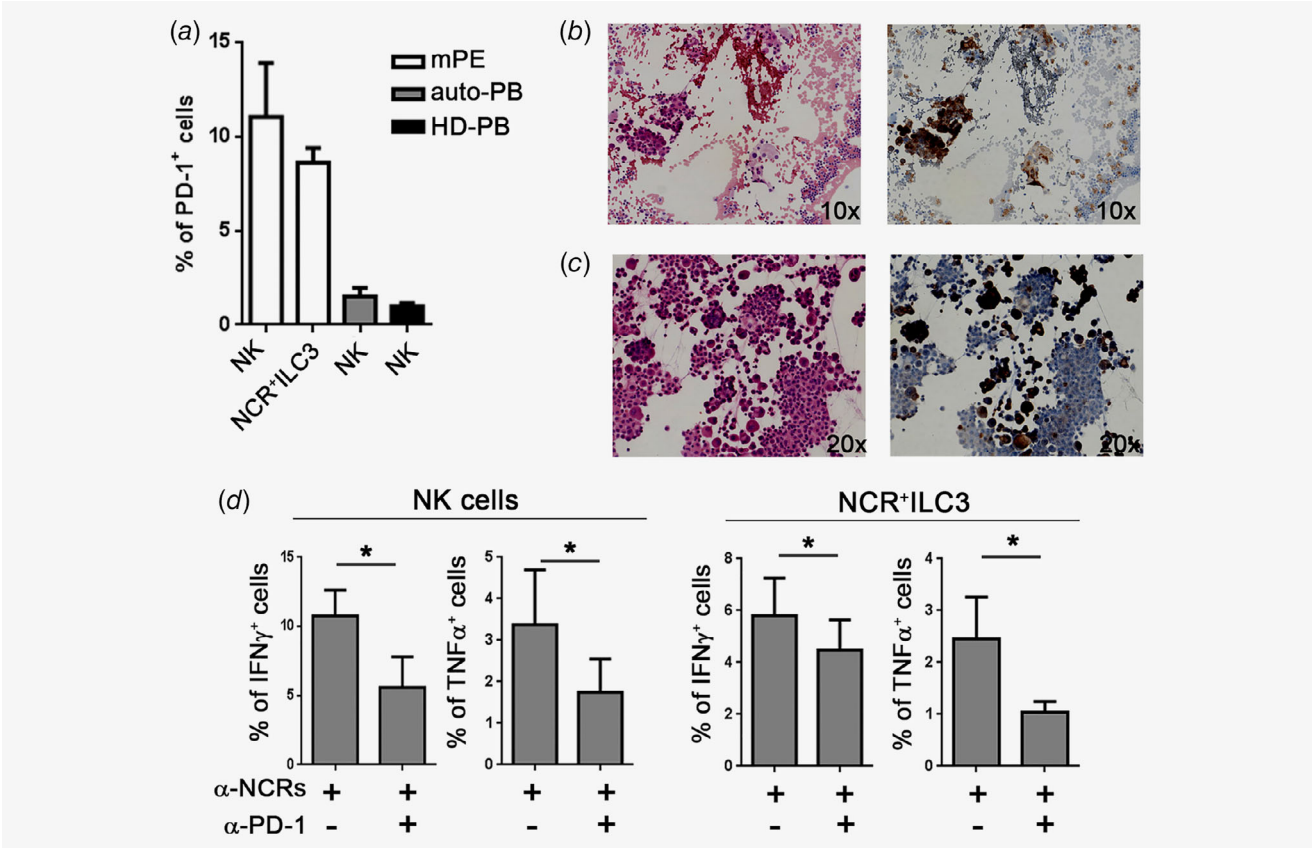

Figure 5. PD-1 checkpoint mediated inhibition of cytokine production by mPE-ILCs. (*a*) Percentage of PD-1$^+$ cells in NK cells or NCR$^+$ILC3 present in mPE (white bars, $n = 12$), in comparison to NK cells isolated from the autologous PB of the same patient (gray bar, $n = 5$) or to NK cells of HD-PB (black bar, $n = 6$), data represents the mean ± SEM. (*b*, *c*) Immunocytochemical analysis of adenocarcinoma PE. Hematoxylin and Eosin (left panels) and PD-L1 expression (brown cells, right panels) in tumor cells. Magnification at (*b*) 10× and (*c*) 20×. (*d*) Cytokine production in the indicated ILC populations upon mAb-mediated cross-linking of NCRs in the absence or in the presence of anti-PD-1 as indicated ($n = 7$). Mean ± SEM of percentage of positive cells. A value of $p < 0.05$ (*) was considered statistically significant. [Color figure can be viewed at wileyonlinelibrary.com]

cytometric analysis revealed that the prevalent ILC subset present both in mPE and in iPE is ILC3. The ILC3 subset includes two subpopulations with different phenotypic and functional features. Based on the expression of NCR and on the pattern of cytokines produced, it is possible to identify NCR$^+$ILC3, that produce mainly IL-22 and IL-8, and NCR$^-$ILC3, that produce IL-17, and TNF-α in mPE. NCR$^+$ILC3 outnumbered NCR$^-$ILC3. The soluble inhibitory factors present in mPE, potentially capable of modulating the antitumor activity of different immune cells, did not appear to substantially affect ILC. Indeed, mPE-ILC could produce cytokines upon short time culture and appropriate stimulation. In particular, ILC3 produced IL-8, IL-22, IL-17 and TNF-α, ILC1 expressed IFN-γ TNF-α, while ILC2 type 2 cytokines, namely IL-13, IL-5 and IL-4. The fact that mPE-ILC2 produce IL-4 (this cytokine is barely detectable in human ILC2 from PB or other sources), may have a pathogenic relevance, in view of the polarizing effect of IL-4 toward type 2 responses which favor tumor growth. In addition, the pro-fibrotic activity of IL-4 may have a further pathogenic effect.

It is well known that, during cancer progression, tumor cells may induce an immunosuppressive state by different mechanisms including the expression of inhibitory checkpoints on immune cells. This could result in inhibition of the antitumor activity and favor tumor immune escape.[53] Notably, clinical efficacy has been well documented in patients with different types of cancer treated with anti-PD-1 mAb.[54] PD-1 expression has been detected in different immune cells, including T, B and myeloid cells while limited information exists on the PD-1 expression on ILC.[38–42] A recent study reported the presence of PD-1$^+$NK cells in the ascitic fluid of ovarian carcinoma, while, the expression of PD-1 on other ILC subsets, particularly in tumors, has not been reported.[46] Our study shows that NK cells and ILC3 present in mPE may express PD-1 and that the size of PD-1$^+$NK cell subset in mPE is much larger than in peripheral blood NK cells, suggesting that the TM may favor PD-1 expression in NK cells. PD-1 expression is mostly confined to mature CD56^{dim} NK cells characterized by a high cytotoxic activity against tumors. Notably, we could detect a reduction of cytokine production by ILC upon PD-1 cross-linking. These data suggest that PD-1 may impair NK/ILC3 mediated responses against tumors that express PD-L1.

In conclusion, our study provides the first evidence that different functional ILC populations are present in mPE.

While their interplay with tumor cells and their influence on either tumor progression or antitumor defenses requires further investigation, our data indicate that ILC and the cytokines that they release may be added to the complex network of cells and soluble factors present in TM. Notably, the relatively easy accessibility to mPE ILC may favor their isolation, expansion *in vitro* and possible use in new protocols of adoptive immunotherapy. In view of the expression of PD-1 on NK cells and on ILC3 and PD-L1 on tumor cells, it is conceivable that the therapeutic use of blocking anti-PD-1 or PD-L1 mAbs in mesothelioma or adenocarcinoma patients with mPE could allow regression of tumor pleural lesions.

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Author Contributions

N.T. and S.M. performed research, interpreted data; E.M. and G.B. performed immunohistochemical analysis; F.S. provided reagents and interpreted data; M.C.M. and L.M. supervised the research and wrote the paper; P.V. designed and performed research, interpreted data and wrote the article.

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