Immunosuppressive Mediators Impair Proinflammatory Innate Lymphoid Cell Function in Human Malignant Melanoma

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Innate lymphoid cells (ILC) are a family of immune cells that are emerging as potent orchestrators of immune responses. In cancer, ILCs display both pro- and antitumorigenic functions depending on the nature of the tumor and the involved ILC subset. Little is known about the ILC–tumor cross-talk in human melanoma. Here, we showed that ILC1s were enriched but functionally impaired in cytokine secretion in both peripheral blood mononuclear cells and tumor-infiltrated lymph nodes of melanoma patients. These findings were confirmed in vivo in murine cutaneous melanoma. Multiple immunosuppressive mechanisms are described in the melanoma microenvironment. Among others, adenosine and kynurenines were shown to suppress antitumor immune responses. By exposing ILCs to adenosine and kynurenines, we observed a similar shift toward the ILC1 subset distribution and impairment in proinflammatory cytokine production to that of patient samples studied ex vivo. Thus, we hypothesized that the immunosuppressive microenvironment of malignant melanoma might shape ILC subpopulations. Hence, we provide a rationale for the use of drugs targeting adenosine and kynurenine pathways in melanoma patients.

Introduction

Immunotherapy holds substantial promise for metastatic melanoma treatment, as demonstrated by the U.S. Food and Drug Administration (FDA) approval of oncolytic viruses that directly kill tumor cells and antibodies that target cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1). Although these treatments significantly increase overall survival, many patients do not respond or develop resistance (1). Experimental and clinical evidence has highlighted the importance of the tumor microenvironment (TME) in promoting cancer development and progression by fostering a local state of immunosuppression (reviewed in ref. 2). The TME is a heterogeneous ecosystem composed of a variety of cancer cells, stroma, and immune cells that engage in an intricate cross-talk, thus shaping antitumor immunity. In particular, there is increasing interest in the immunosuppressive circuits established within the TME, including the upregulation of indoleamine-2,3-dioxygenase 1 (IDO or IDO1) and the accumulation of adenosine (ADO ref. 3, 4). IDO1 is the key rate-limiting enzyme involved in the regulation of tryptophan catabolism, resulting in the production of L-kynurenine (reviewed in ref. 5). IDO1 activity inhibits the proliferation and activation of immune cells in the TME; in fact, tryptophan starvation and high kynurenines synergistically drive CD8+ T cells, CD4+ T cells, and natural killer (NK) cells to undergo cell-cycle arrest and functional anergy, and reduce the immunogenicity of dendritic cells (DC), leading to a tolerant state that further suppresses antitumor immune responses (6–9). The IOD1 inhibitor, indoximod, in combination with the anti–PD-1, pembrolizumab, increases the overall response rate (ORR) of advanced-melanoma patients in a phase II clinical trial (10); however, no survival benefit was shown in the phase III ECHO-301 trial that combined the IOD1 inhibitor epacadostat and pembrolizumab, suggesting the need for better designed IOD1-targeting trials (11).

ADO is another potent immunosuppressive metabolite that is mainly produced by the hydrolysis of ATP. This is mediated by the ectoenzyme CD39, which converts ATP to ADP and AMP, and by the rate-limiting ectonucleotidase CD73, which transforms ADP/AMP into adenosine (12). ADO’s immunosuppressive effects are mainly exerted via activation of G protein–coupled adenosine receptors (ADORA), such as A2A and A2B, which are expressed in several immune cell populations, including CD4+ T cells, CD8+ T cells, and NK cells (13–15). Monteiro and colleagues characterized CD73 expression in human metastatic melanoma and in tumor-infiltrating mononuclear cells, supporting the idea that the use of anti-CD73 antibodies or A2AR inhibitors can enhance the antitumor activity of both PD-1 and CTLA-4 blocking antibodies (16). This idea is further demonstrated by various clinical studies focusing on targeting adenosine in solid tumors (4, 16, 17).

Innate lymphoid cells (ILC) contribute to the complex cell network within the TME by directly impacting the balance of antitumor immune responses (reviewed in refs. 18–20). ILCs are lineage-negative cells (as described in the Material and Methods section) constitutively expressing CD127 (IL-7Rα chain). They are divided into...
Materials and Methods

Human cell collection

Venous blood was drawn from 48 unselected healthy, routine donors at the blood transfusion center of Lausanne, approved by the Human Research Ethics Committee of the Canton of Vaud, Switzerland. All subjects gave their written consent. PBMCs were isolated by Lymphoprep centrifugation (1,800 rpm, 20 minutes centrifugation without break, room temperature), washed twice and immediately cryopreserved in 90% fetal calf serum (FCS) and 10% DMSO. Peripheral blood (PB) from 41 stage III/IV malignant melanoma patients (median age 47; range, 24–80) was obtained from the Department of Oncology, University Hospital (CHUV), Lausanne, Switzerland (NCT00112242), under approval of the Lausanne University Hospital’s Institute Review Board. PBMCs were isolated at Lausanne branch in 1996 to 1997 from metastases excised from patients LAU50 and LAU203. They have been maintained since 2000 and maintained by serial passage in RPMI-1640 (Eurobio) supplemented with 1% HEPES, 1% streptopencillin, and 10% FCS.

ILC evaluation by flow cytometry

Human ILCs were identified as lineage (Lin)-negative and CD127-positive cells. Lineage markers, all FITC conjugated, include anti-human CD3 [UCHT1, Beckman Coulter (BC)], anti-human CD4 (SFC124D11, BC), anti-human CD8 (MEM-31, Immunotools), anti-human CD14 (RMO52, BC), anti-human CD16 (3G8, BC), anti-human CD15 (80H5, BC), anti-human CD19 (J3-119, BC), anti-human CD20 (2H7, BioLegend), anti-human CD33 (HIM3-4, BioLegend), anti-human CD127 (IL-7Rα, BioLegend) or PE Dazzle anti-human CD127 (IL-7Rα; A019D5, BioLegend), PE anti-human CD117 (cKit; YB5.B8, BD Biosciences), PE anti-human CRTH2 (CD294; BM16, BioLegend) or Brilliant Violet 421 anti-human CRTH2 (CD294; BM16, BD Biosciences) or PCF594 anti-human CRTH2 (CD294; BM16, BD Biosciences), PerCPCy5.5 anti-human CD335 (NKp46, 9E2, BD Biosciences), or PCeCy7 anti-human CD335 (NKp46, 9E2, BD Biosciences). Cells were stained for 20 minutes at room temperature in 50 μL of FACS buffer. Dead cells were excluded using the viability dye Live/Dead Aqua or Vivid IR (Invitrogen) diluted in 200 μL of PBS for 30 minutes at 4°C. Where indicated, additional markers were evaluated using PerCPCy5.5 anti-human CD14 (HC1D4, BioLegend), PECy7 anti-human CD39 (A1, BioLegend), and PCF594 anti-human CD73 (AD2, BD Biosciences). Intracellular staining was performed after fixation and permeabilization with 0.1% saponin (Sigma), using APC anti-human IL13 (JES-10-5A2, BD Biosciences), PerCPCy5.5 anti-human IL13 (JES10-5A2, BioLegend), PeCy7 anti-human IFNγ (4S, B3, BD Biosciences), Alexa Fluor 700 anti-human IL17A (BL168, BioLegend), PerCPCy5.5 anti-human TNFx (MAB11, BioLegend), APC anti-human TNFx (MAB11, BD Biosciences), PE anti-human IgG1κ isotype (MOPC-21, BioLegend), PE anti-human IDO (eyedio, eBioSciences), Alexa Fluor 647 anti-human IDO (2E2/IDO1, BioLegend), and Brilliant Violet 421 anti-human ILS (TREK5, BioLegend). Samples were acquired on a Gallios flow cytometer (Beckman Coulter) or LSRFortessa (BD), and data were analyzed using FlowJo software (TreeStar V10). For ILC isolation, we used the same panel described above for ILCs identification, aliquots of cells were sorted to 98% purity using a FACSria (Becton Dickinson), based on the gating strategy illustrated in Fig. 1.

Murine ILCs were identified as CD45+ Lin- CD90.2+ among live cells. Lineage markers, all FITC conjugated, include anti-mouse CD3e (17A2, in house), CD5 (53.7, in house), CD19 (ID3, in house), CD11b (M1/70, in house), CD11c (N418, in house), B220 (RA3-6B2, in house), CD49b (DX5, Miltenyi Biotec), FcεRIt (MAP-1, Miltenyi Biotec), Ter119 (Ter119, in house), TCRβ (2M31/11, in house), and TCRβ (H57, in house). Additional markers used to identify the ILC subpopulations include Alexa Fluor 700 anti-mouse CD45.2 (AL1-4A2, in house), PCeCy7 anti-mouse CD127 (A7R34, eBioSciences), APC-Cy7 anti-mouse CD117 (cKit; 2B8, eBioSciences), and PE anti-mouse ST2 (RMST2-2, Invitrogen).

Cell culture

The murine melanoma cell line B16-F10 was obtained from the laboratory of Dr. Lars French, University Hospital of Geneva, in 2000 and maintained by serial in vitro passages from cryopreserved stocks in DMEM supplemented with 1% HEPES, 1% streptopenicillin, and 10% FCS. The human Me290 and Me275 melanoma cell lines were established at the Ludwig Institute for Cancer Research, Lausanne branch in 1996 to 1997 from metastases excised from patients LAU50 and LAU203. They have been maintained since then by serial in vitro culture passages, from a master cell bank stock preserved at −110°C (23). Cells were not authenticated in the past year. Cells were cultured for approximately 3 to 4 weeks in RPMI-1640 (Eurobio) supplemented with 10% heat-inactivated fetal bovine serum (Eurobio), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.01 M HEPES buffer and 1 mmol/L sodium pyruvate (Eurobio) and were regularly tested for Mycoplasma contamination by PCR. Cells were grown until 75% confluence and passed using trypsin/1× EDTA (Eurobio), washed with PBS and resuspended in supplemented RPMI-1640. Human PBMCs were cultured in RPMI, supplemented with 8% heat-inactivated pooled human serum, in the presence of 20 U/mL rhIL2 (Proleukin, Roche). Cells were grown at 37°C in a humidified incubator under 5% CO2. When indicated, D-kyurenine (diluted...
in HCl), L-tryptophan, C-adenosine (Cado), 1-methyl-L-Tryptophan, and ZM241385 (all diluted in DMSO and from Sigma-Aldrich) were added at the indicated concentrations. For the evaluation of cytokine production, cells were stimulated with 1 μg/mL PMA plus 0.5 μg/mL ionomycin (P1585 and I9657, respectively from Sigma-Aldrich), in the presence of brefeldinA (B6542, Sigma-Aldrich) for 3 hours prior to intracellular staining.

**Generation of an IDO-expressing melanoma cell line**

Human IDO-1 (UniProt P14902) was cloned in a pMSGV retroviral vector downstream of the blasticidin resistance gene followed by a P2A element. Viral particles were produced by mixing in 250 μL of Optitenn medium (Life Technologies) pMSGV (1.25 μg) and packaging plasmids pMD.gagpol (1.25 μg) and pMD.G (1.25 μg, VSV-G envelope protein) with 7.5 μL of MIRUS reagent (Mirus Bio LLC). After 20 minutes at room temperature, the mix was added to 1 million of 293T cells. After 48 hours, 5 mL of virus-containing supernatant was collected and added to Me275 melanoma cells at 50% of confluence in 25 mm2 flasks. The following day, the virus was removed, and blasticidin (15205, Sigma-Aldrich) was added to the culture medium at a concentration of 500 μg/mL until selection of a pure population of cells expressing IDO was established. IDO expression and function were determined, respectively, by flow cytometry staining using an anti-IDO antibody (clone 2E2, BioLegend) and by Erhlich test (Sigma-Aldrich) following the manufacturer’s instruction.

**Cutaneous melanoma model in C57BL/6 mice**

Female C57BL/6J mice, 6 weeks old and weighing 18 to 20 g, were purchased from Envigo. B16-F10 murine melanoma cells (1 × 10^5) in 100 μL saline were injected subcutaneously (s.c.) into the right flank of C57BL/6 mice. Mice were observed daily and humanely euthanized by CO₂ inhalation 21 days after injection; tumor and draining LNs were then collected. Tumors were minced into small pieces in C-tubes followed by mechanical dissociation using the tumor02 program in the gentleMACS dissociator (Miltenyi Biotec). Samples were digested using the Mouse Tumor Dissociating kit (130-096-730, from Miltenyi Biotec) according to the manufacturer’s recommendations. After the last mechanical disruption step, the digested tumors were harvested, filtered using a 70-μm strainer, and the lymphocyte fraction enriched using a 40% to 70% Percoll gradient (1,800 rpm, 20-minute centrifugation without break, room temperature). Draining LNs were harvested, dissociated mechanically with the piston of a syringe directly into the 40-μm strainer. Experiments were performed in accordance with Swiss ethical guidelines and with the protocols described in the animal license VD-3255.a.

**In vitro coculture experiments**

PBMCs from HDs were cultured with the Me290 melanoma cell line in a ratio of 10:1 (1 melanoma cell: 10 PBMCs) in RPMI complete medium. After 48 hours, PBMCs were harvested and analyzed to detect ILC frequencies and cytokine production by flow cytometry analysis.

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**Figure 1.**

ILCs were significantly increased in human melanoma. **A**, Representative examples of flow cytometry analysis of ILCs in HD and melanoma patients’ PBMCs. **B**, Frequencies (HD n = 48 and melanoma n = 41) of total ILCs identified as Lin−CD127+ cells in lymphocytes. **C**, Representative examples of flow cytometry analysis of ILC subsets in HD and melanoma patients’ PB. **D**, Frequencies of ILC subsets among total ILCs. **E**, Frequencies of total ILCs in melanoma patients’ NLN and TILN. **F**, Frequencies (NLN n = 6 and TILN n = 19) of ILC subsets among total ILCs. **G**, Frequencies of cytokine production by ILCs from ex vivo HD PBMCs, melanoma patients’ PBMCs, and NLN or TILN (HD PBMCs n = 9, melanoma PBMCs n = 6, NLN n = 5, and TILN n = 5). In all box charts, data, mean ± SEM (*, P < 0.05; ****, P < 0.0001). Wilcoxon and/or two-way ANOVA tests.
IDO activity assay

The amount of L-kynurenine in culture supernatants was measured by spectrophotometric analysis, as previously described (24, 25). Cells (either the Me290 line or magnetically purified human CD14+ monocytes; Miltenyi Biotec) were pre-stimulated with 1,000 U/mL human IFNγ (PeproTech) or 50 ng/mL human TNFα (PeproTech) for 24 hours. Next, cells were washed and resuspended in Hanks buffered saline solution containing 500 µM/L L-tryptophan (Sigma-Aldrich) and incubated at 37 °C for 4 hours. Supernatants were then harvested and mixed with 30% trichloroacetic acid. After centrifugation at 8,000 × g for 5 minutes, the solution was added in a ratio of 1:1 with 4-(dimethylamino) benzaldehyde (Ehrlich reagent, Sigma-Aldrich) in a 96-flat bottom well plate. Optical density was measured at 490 nm, using a BioTek Epoch Microplate Spectrophotometer. Triplicate samples were run against a standard curve of defined kynurenine concentrations (0–1,000 µM/L; Sigma-Aldrich).

RNA purification and quantitative real-time PCR

Total RNA was isolated from highly pure, sorted human ILC subsets using the TRizol reagent according to the manufacturer's instructions (15596026; Invitrogen). Final preparation of RNA was considered DNA- and protein-free if the ratio of readings at 260/280 nm was ≥ 1.7 after quantification with nanodrop. Total RNA (1 µg) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems) according to the manufacturer's protocol. The quantitative real-time PCR (qPCR) was carried out in the Applied Biosystems 7900HT Fast Real-Time PCR Sequence Detection System (Applied Biosystems) with specific primers (hA1 5'-CCTCCATCTCAGCTTCCAG-3', 5'-AGTAGGTCTTGTGGGCCAATG-3', hA2A 5'-CTCCGGTACAAAGGGCTTTG-3', 5'-CTGTTTCGTGCGCTCTTTGGG-3'; hA2B 5'-ATGCCCAACAGC-TTGATAGGAT-3', 5'-GAGGTCACCTTGCTTCGGAC-3'; hA3 5'-TTGACCAAAAGGAGGAGAAGT-3', 5'-TTGAATGGAT-3'). The expression of CD117 (cKit) and CRTH2 (CD294) surface markers was assessed in the LNs obtained from melanoma patients during LN dissection surgery. Based on routine histologic assessment, these LNs were classified as TILN and non–tumor-infiltrated LN (NLN). An increase in the frequency of total ILCs was found in TILN when compared with NLN, although this was not statistically significant (Fig. 1E). However, similar to the findings in the PBMC, we observed a significant increase in ILC1s in TILN compared with NLN (Fig. 1F). Next, we assessed the functionality of ILCs by evaluating the cytokine production in PBMCs from HDs and in PBMCs and TILN from melanoma patients after ex vivo stimulation. ILCs from PBMC and TILN of melanoma patients were substantially impaired in IFNγ production. This reduction was accompanied by a trend in the increase in the production of Th2-associated cytokines in PBMCs from melanoma patients (i.e., IL5 and/or IL13; Fig. 1G). Collectively, these results suggested that ILC frequency, subset, and function were dysregulated in melanoma patients. In particular, ILC1s were enriched in both PBMCs and TILN and had impaired IFNγ production.

ILCs were enriched in tumor and LN of B16-F10 melanoma–bearing mice

To investigate the role of ILCs in melanoma development, we used the most widely acknowledged experimental model to study melanoma in vivo (26), the subcutaneous B16-F10 murine melanoma model. Analysis of LN from tumor-bearing mice compared with control mice did not show any difference in the frequency of total ILCs (Supplementary Fig. S1A and S1B). However, similar to the results obtained in human samples, ILC1s were enriched in both TILN and dissociated tumor (Supplementary Fig. S1C–S1E).

Coculture of ILCs with human melanoma cells mimicked observations made in patients

To extend our evaluation to mechanistic analyses, an in vitro coculture assay was performed. In-house–generated Me290 melanoma cells were plated with fresh PBMCs from HDs in a ratio of 1:10 and incubated for 48 hours to evaluate ILC frequency and function by flow cytometry. Exposure of HD PBMCs to the Me290 melanoma cell line induced the reduction of total ILCs (Fig. 2A and B). Quantifying the distinct ILC subsets, we found a significant enrichment of ILC1s at the expense of ILC3s (Fig. 2C and D). In addition, we also assessed IFNγ and TNFα secretion. As shown in Fig. 2E, ILCs cocultured with the Me290 melanoma cells had significantly reduced IFNγ production. Collectively, these data suggest that the contact between ILCs and melanoma cell lines induced the change in ILC frequency and function as observed in melanoma patients ex vivo.

Kynurenine- and adenosine-producing enzymes were expressed in Me290 melanoma cells

IDO1 upregulation accompanied by kynurenine production and ADO production are two well-known immunosuppressive mechanisms in malignant melanoma (27–30). We thus investigated the possible involvement of these two mediators in the observed ILC dysfunction. First, we characterized the Me290 melanoma cell line for the expression of IDO1 and two of the adenosine-producing enzymes, CD39 and CD73. Because IDO1 can be induced by proinflammatory
signals in both healthy and tumor cells (31), Me290 cells were incubated for 24 hours in the presence of IFN\(\gamma\) and TNF\(\alpha\) prior to the evaluation of IDO1 expression by flow cytometry (Fig. 3A). Purified CD14\(^+\) monocytes stimulated with IFN\(\gamma\) were used as a positive control. As summarized in Fig. 3B, Me290 melanoma cells do not express IDO1 in their resting condition. By contrast, incubation of Me290 melanoma cells with IFN\(\gamma\) and TNF\(\alpha\) significantly induced IDO1 expression and activity (Fig. 3C) and stimulated cells were able to produce kynurenines in the presence of L-tryptophan. This production was reversed by the addition of the IDO1-specific inhibitor 1-MT-L (Fig. 3D). In addition, we assessed the expression of CD39 and CD73 by the Me290 melanoma cell line by flow cytometry analysis (Fig. 3E). Me290 cells constitutively express CD39, whereas only 10% to 20% of Me290 cells express CD73 (Fig. 3F and G). Lastly, we also evaluated IDO1, CD39, and CD73 expression on human ILCs (Supplementary Fig. S2). Low IDO1 expression could be detected in all ILC populations (Supplementary Fig. S2A and S2B). As opposed to the observations made in the Me290 cell line, ILCs, particularly ILC1s, expressed high CD73 and low CD39 (Supplementary Fig. S2C and S2D). These findings suggested that the IDO1 and ADO pathways were active in melanoma cells and that combined with the presence of CD73 on ILCs, this might further contribute to sustaining the ADO–immunosuppressive axis.

Impact of kynurenines and adenosine on ILC phenotype and function

In order to determine the effect of kynurenines on ILC subtype composition and function, fresh PBMCs were incubated with L-kynurenines for 48 hours and then stained for ILC subset phenotypic markers. Although not statistically significant, we observed a trend increase in the ILC1 population after treatment with kynurenines compared with the control. No differences were observed in ILC2 and ILC3 populations (Fig. 4A). Next, we investigated whether L-kynurenines could affect ILC function in terms of cytokine production. PBMCs were incubated with L-kynurenine for 48 hours, stimulated with PMA/ionomycin and then stained for IFN\(\gamma\) and TNF\(\alpha\) production. As shown in Fig. 4B and C, IFN\(\gamma\) production was completely abrogated and TNF\(\alpha\) significantly reduced in the presence of kynurenines. Similar observations were obtained using a melanoma cell line transduced with a lentiviral vector expressing the full-length IDO protein (Supplementary Fig. S3). Next, fresh PBMCs were cultured with Cado, a stable form of adenosine, to assess ILC frequency and function after overnight incubation. Similar to the kynurenine treatment, we did not find any difference in ILC frequency and subset distribution (Fig. 4D). However, treatment with C-adenosine significantly reduced IFN\(\gamma\) production, whereas only a minor decrease in TNF\(\alpha\) production was observed (Fig. 4E and F). These results suggested that kynurenines and adenosine produced by melanoma cells may directly contribute to the phenotypic and functional ILC shift observed in patients.

The ADO-mediated inhibitory effects on ILCs were partially mediated by the A2A receptor

Given the remarkable interest in targeting the adenosine pathway in melanoma clinical trials (17) and based on our findings, we sought to characterize the expression of the ADORA receptors on ex vivo sorted total ILCs from HD PBMCs. As expected, ADORA receptors were prominently expressed in total ILCs (except for A3 receptor). The A2A receptor was the most highly expressed followed by comparable expression of A1 and A2B receptors (Fig. 5A). To address the functional importance of the A2B receptor in ILCs, we analyzed the effect of the A2A-selective antagonist ZM241385 on cytokine
production. IFNγ production was consistently upregulated by A2A antagonism (Fig. 5B). These findings suggest that the adenosine-mediated inhibitory effects on ILCs were, at least in part, governed by the A2A receptor.

**Discussion**

Despite advances in our knowledge of ILC biology (e.g., subset distribution and plasticity), the role of these cells in cancer, especially in patients, remains poorly characterized (reviewed in refs. 19, 20, 32). In this study, we characterized ILCs in human melanoma and observed an enrichment of ILC1s, accompanied by an impairment in their proinflammatory cytokine secretion. Previous work from our group and others report expanded, but dysfunctional ILC1s in different types of human hematologic malignancies (19, 33–35). Whether this is also a common hallmark of human solid cancers remains to be confirmed.

The hypothesis of ILC1 dysfunction in malignancy is supported by the observation that unconventional type 1–like ILCs, together with type 1 innate–like T cells, locally ensure immune surveillance in oncogene-driven murine prostate and breast cancer models (36). Conversely, two independent studies show that TGFβ in the TME can convert NK cells into ILC1-like populations, which are unable to control tumor growth and metastasis (37, 38). Further work is needed to identify the human correlates of these observations.

Melanoma progression and resistance to therapy are promoted by the presence of a potent immunosuppressive microenvironment, including both neoplastic and nonneoplastic cells (reviewed in refs. 39, 40). Similar to the well-known immunosuppressive effect of the TME on adaptive immune cells, complex mechanism(s) might also modulate the distribution of ILC populations and their function. This complex network is at least partly dependent on the presence of different soluble factors, such as cytokines, vitamins, amino acids, and lipids, present in the tumor milieu. Interestingly, some of these mediators directly modulate ILC polarization in non-tumor settings (41–43). Based on this, we hypothesized that the immunosuppressive mechanisms described in the TME of melanoma patients could be involved in the shifts observed in ILC subset composition and impaired ILC1 function. IDO1 is broadly activated in human cancer, including malignant melanoma, in an IFNγ-dependent manner (44, 45). However, although the suppressive effect of kynurenine accumulation has been largely studied on T and NK cells in the TME (46), there have been no reports of its impact on ILCs. Kynurenine is a potent agonist of the Aryl hydrocarbon receptor (AHR), a transcription factor expressed by ILC3s/ILCPs (47). In turn, AHR positively regulates IDO1 expression and subsequent kynurenine production (8). Despite the low expression of IDO1 observed in ILCs, we cannot exclude that, in the TME, kynurenine may have induced IDO1 in ILCs, resulting in an AHR/kynurenine/IDO1-positive feedback loop. Moreover, in T cells, AHR activation regulates type 1 regulatory T (Tr1) cell generation. Whether ILC3s/ILCPs may become a regulatory subset upon AHR activation by kynurenine in the TME needs addressing (48). Our ex vivo and in vitro data support a direct role of kynurenines in functional ILC impairment in melanoma, which is of particular interest in the context of current immunotherapy trials targeting IDO1 with selective inhibitors. It would be highly informative if biomarker analyses on samples from the ECHO-301 trial included ILC profiling (11). Moreover, the assessment of expression of the other tryptophan catabolic enzymes IDO2 and TDO in tumor-
infiltrating ILCs might unravel potential compensatory mechanisms operating during immunotherapy with selective IDO1 inhibitors. Understanding the impact of IDO/TDO biology on innate lymphocytes may provide new targets in the blockade of tryptophan catabolism in cancer.

The adenosinergic signaling axis has emerged as a powerful immune checkpoint in the TME (17). Elevated ATP is observed in different tumors (reviewed in ref. 49), and ATP catabolism is primarily mediated by CD39 and CD73 (50). Whereas the expression of these two ectoenzymes has been reported on several immune and stromal cell types, their expression on ILCs remains unknown. Our observation that CD73 was detected on ILCs advocates for a contribution of these cells in the generation of ADO, a metabolite known to potently restrict immune responses via pleiotropic effects in the TME (51). Besides participating in ADO generation, ILCs might also be directly inhibited by ADO. We observed expression of the high-affinity A2A receptor on human ILCs, which is one of the 4 known ADO receptors. ADO signaling through A2A and A2B receptors is the major pathway mediating the ADO-immunosuppressive effect in immune cells (52, 53). We speculated that the reduction of IFNγ production by ILCs after ADO treatment was mediated by the activation of the A2A receptor, as previously shown for T and NK cells (54, 55). Using the selective A2A receptor antagonist ZM241385, we confirmed significant upregulation of IFNγ production. Thus, our results indicated that the reduced production of IFNγ is partially mediated by ADO through the A2A receptor. Further work will be needed to determine the exact impact of ADO on ILCs as opposed to its action on their adaptive counterparts in antimelanoma responses.

In conclusion, we have demonstrated that ILCs were dysregulated in human melanoma, which was due, at least in part, to the presence of kynurenines and adenosine in the TME. We also demonstrated that the inhibition of one of the adenosine receptors reverts the production of IFNγ. In the current milieu of promising preclinical and clinical approaches targeting the IDO and adenosinergic immunosuppressive axes, we have generated new evidences to support blocking these pathways in melanoma patients.

Figure 4.
Impact of kynurenines and adenosine on ILCs. A, Frequencies of ILC subsets among total ILCs after L-kynurenine treatment 480 μmol/L for 36 hours (HCl n = 8; L-kynurenine n = 9). B, Representative example of flow cytometry analysis of ILCs upon treatment with L-kynurenine (L-kyn). C, Frequencies of IFNγ+ and TNFα+ in total ILCs after treatment for 36 hours with L-kynurenine 480 μmol/L (n = 6). D, Frequencies of ILC subsets among total ILCs after overnight treatment with Cado 25 μmol/L (n = 6). E, Representative example of flow cytometry analysis of ILCs upon treatment with Cado. F, Frequencies of IFNγ+ and TNFα+ in total ILCs after overnight treatment with Cado 25 μmol/L (n = 6). In all box charts, data, mean ± SEM (***, P < 0.001; ****, P < 0.0001). Two-way ANOVA tests.

Figure 5.
ADO inhibitory effects were in part mediated by the A2A receptor. A, Expression of A1, A2A, A2B, and A3 adenosine receptors assessed by qPCR in freshly sorted total (TOT) ILCs (n = 5). B, Frequencies of IFNγ+ production in total ILCs after overnight treatment with Cado or Cado/2M241385 (Cado + 2M) 20 μmol/L (n = 6). In all box charts, data, mean ± SEM (*, P < 0.05). Wilcoxon and one-way ANOVA tests.
Disclosure of Potential Conflicts of Interest
P. Romero is a consultant/advisory board member for Immatics Biotechnologies, reports receiving other commercial research support from Roche pREID, Zurich, and reports receiving speakers bureau honoraria from Bristol-Myers Squibb, AstraZeneca, and Roche. No potential conflicts of interest were disclosed by the other authors.

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References


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