IL13-Mediated Dectin-1 and Mannose Receptor Overexpression Promotes Macrophage Antitumor Activities through Recognition of Sialylated Tumor Cells

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Abstract

Macrophage-mediated cytotoxicity is controlled by surface receptor expression and activation. Despite the numerous studies documenting the role of macrophage C-type lectin receptors (CLR) in pathogen elimination, little is known about their contribution to antitumor responses. Here, we report that IL13 inhibits T-cell lymphoma and ovarian adenocarcinoma development in tumor-bearing mice through the conversion of tumor-supporting macrophages to cytotoxic effectors, characterized by a CLR signature composed of dectin-1 and mannose receptor (MR). We show that dectin-1 and MR are critical for the recognition of tumor cells through sialic acid–specific glycan structure on their surface and for the subsequent activation of macrophage tumoricidal response. Finally, we validated that IL13 antitumor effect mediated by dectin-1 and MR overexpression on macrophages can extend to various types of human tumors. Therefore, these results identify these CLRs as potential targets to promote macrophage antitumor response and represent an attractive approach to elicit tumor-associated macrophage tumoricidal properties.

Introduction

Monocytes/macrophages represent a major component of leukocyte infiltration in tumors. The beneficial and adverse effects of macrophages on tumor progression result from their great heterogeneity and plasticity (1–3). Within the tumor, macrophages are commonly termed tumor-associated macrophages (TAMs). During early-stages tumor development, tumor-infiltrating macrophages, which promote immune responses and elicit tumor cell disruption, are characterized by an IL12high and IL10low phenotype, close to M1-polarized responses and elicit tumor cell disruption, are characterized by a CLR signature composed of dectin-1 and mannose receptor (MR). We and tumor- associated macrophage tumoricidal activity (4). In late-stage tumor progression, TAMs switch to an IL12low and IL10high phenotype with low tumoricidal activity (5, 6). Although most reports identify them as anti-inflammatory M2-like macrophages, activated TAMs produce multiple proinflammatory markers characteristic of M1 phenotype (3, 7). Thus, the subset terminology M1 and M2 is oversimplified and does not accurately reflect the subpopulations of TAMs (4, 8).

Several lines of evidence suggest that the acquisition of cytotoxic functions in macrophages depends on the expression and activation of pattern recognition receptors (PRR). The stimulation of Toll-like receptors (TLR) on macrophages promotes the activation of cytotoxic pathways, shifting tumor-supporting macrophages toward tumoricidal effectors (9, 10). Likewise, within the PRR family, C-type lectin receptors (CLR) can also modulate antitumor responses, because they can specifically bind the tumor-related glycoforms and can affect CLR signaling and immune cell differentiation (11). The CLRs DC-SIGN and MGL expressed on dendritic cells (DC) can interact with altered glycosylation patterns found on tumor cells and impair DC function, thereby preventing optimal priming of tumor-specific T cells (11–14). Conversely, myeloid CLRs can deliver an activating signal to NK cells or lymphocytes in response to detection of altered self and participate to tumor elimination. This was reported for the NKG2D receptor, a CLR expressed by NK cells that mediates the generation of CD8+ cytotoxic lymphocytes and induces CD4+ T helper antitumor responses (15). Similarly, dectin-1 on the surface of macrophages is important in the recognition of tumor cells that highly express glycan structures and in the activation of NK...
cell–mediated tumor cell killing (16). Thus, myeloid CLRs have relevant and divergent roles in tumor development.

Because the IL13-activated macrophage subset is characterized by abundant amounts of CLRs (4, 8), they can recognize tumor cells and activate signaling pathways leading to antitumor functions. Here, we report that IL13 exhibited antitumor activity against T-cell lymphoma and ovarian adenocarcinoma through reactive oxygen species (ROS) release and arginase-induced L-arginine depletion. We also showed that the antitumor response of IL13-activated macrophages was dependent upon the recognition of sialic acid epitopes on the surface of tumor cells by MR and dectin-1. Thus, these results, which identify these CLRs as a major component of the antitumor response of macrophages, represent an attractive target with which to promote tumoricidal activity of macrophages and may offer new insight into TAM-targeting anticancer treatment.

**Materials and Methods**

**Cell culture**

A murine T-lymphoma cell line (EL4-luc2) was purchased from Caliper in 2014 (Caliper Life Sciences). Human T-cell leukemia cells (Jurkat) were provided by Professor B. Segui in 2014 (CRCR, Toulouse, France). Murine and human ovarian adenocarcinoma cells (ID8, Skov-3) and human breast adenocarcinoma cells (MDA-MB-231), provided by Professor B. Couderc in 2015 (CRCR, Toulouse, France), were transfected with a vector expressing firefly luciferase (17) for bioluminescence quantification. Cells were maintained under limited passage from original stocks (typically under 10). All cell lines were tested negative for Mycoplasma (Plasmotest; Invivogen).

**Mice**

All mouse experiments were performed according to protocols approved by the ethical committee for animal experiments of Midi-Pyrénées, France (Experimentation permit number 31-067) in accordance with the European legal and institutional guidelines (2010/63/UE) for the care and use of laboratory animals (approval number C 31 555 03). B6, Tfla−/−, Tnf−/−, Stat6−/−, and their corresponding controls were purchased from the Jackson Laboratory or Janvier Labs. Clec7a−/−, Mrc1−/−, and Pparg−/− mice have been described earlier, and the corresponding floxed littermates were used as controls throughout all the experiments (18, 19).

**Preparation of mouse resident peritoneal macrophages**

Resident peritoneal cells were harvested from the murine peritoneal cavity with NaCl 0.9%. Collected cells were centrifuged, and cell pellet was suspended in DMEM supplemented with L-glutamine, penicillin, streptomycin, and 5% heat-inactivated fetal calf serum. Cells were allowed to adhere for 2 hours at 37°C and 5% CO2. Nonadherent cells were then removed by several washes with PBS. After 2 hours of adhesion, >95% of adherent cells were macrophages as determined by F4/80 staining. The 5% of contaminating cells are B cells as determined by CD19 staining. Adherent murine peritoneal macrophages were pretreated or not 24 hours with IL13 (Clinsciences, 50 ng/mL), IL4 (Clinsciences, 50 ng/mL), IFNγ (Invitrogen, 50 ng/mL), TNFα (Invitrogen, 50 ng/mL), IL10 (PeproTech, 50 ng/mL), IL6 (PeproTech, 50 ng/mL), or LPS (Sigma-Aldrich, 100 ng/mL) before tumor cell challenge. Thirty minutes before tumor cell challenge and during the coculture period with tumor cells, macrophages were treated or not with Z-VAD-FMK (Z-vad; Calbiochem, 50 μmol/L), necrostatin-1 (Sigma, 1 μmol/L), N-acetyl cysteine (NAC; Sigma, 5 mmol/L), L(+)-arginine (BDH ProLabo, 1 mmol/L), sialic acid (Sigma, 100 μg/mL), Lac-Nac (Sigma, 0.0001–100 μg/mL), Sia-Lac-Nac (Sigma, 0.0001–100 μg/mL), Bay 61-3606 (Sigma, 10 μmol/L), MAFP (Sigma, 10 μmol/L), or 15(S)-HETE (Cayman, 1 μmol/L). For all in vitro experiments, the tumor cell-to-macroage ratio used was 1:10.

**Preparation of human monocyte-derived macrophages (MDMs) and si-RNA gene silencing**

Monocytes were obtained from healthy blood donors (Etablissement Français de Sang; EFS). Written informed consents were obtained from the donors under EFS contract number 21/IVNT/TOU/IPBS01/2009-0052. According to articles L1243-4 and R1243-61 of the French Public Health Code, the contract was approved by the French Ministry of Science and Technology (agreement number AC 2009-921).

Monocytes were differentiated into IL13-MDMs by treatment for 72 hours with IL13 (Clinisciences, 50 ng/mL). To silence dectin-1 and MR into MDMs, lipid-based siRNA-mediated gene silencing was performed as previously described (19). Briefly, siRNA/lipid complexes are added dropwise onto MDMs. Each well contains a siRNA final concentration of 200 nmol/L and 3.0% (vol/vol) of HiPerFect transfection reagent (Qiagen). As soon as the complexes were added to all wells, the plates were incubated at 37°C and 5% CO2 for 6 hours. After transfection time, the reactions were stopped by adding medium with the presence of IL13 (50 ng/mL).

**Extraction of T cells and quantification of extracellular membrane carbohydrates**

Mouse T cells were extracted from spleen using a negative selection Mouse T-cell enrichment kit (Stemcell), as recommended by the manufacturer’s protocol.

To analyze carbohydrate composition at the surface of T lymphocytes and tumor cells, the plasma membrane of cells was recovered from total cell lysate by performing several centrifugation steps. Cell envelopes were suspended in 200 μL buffer and 10 μL of PNS Caso solution (50 U/100 μL water) were added. The incubation occurred at 37°C for 3 hours. The cell envelopes were then removed by centrifugation at 3500 rpm for 10 minutes. Supernatant was freeze-dried and submitted to a 3N MeOH/HCl (Aldrich) hydrolysis (100 μL at 80°C for 1 hour). Fatty acids were then extracted from the medium with chloroform (100 μL). Saccharide acetylation was performed with 50 μL acetic anhydride (Fluka) and 5 μL pyridine (Fluka). After complete evaporation, the sample was dissolved in acetonitrile (50 μL). Various monosaccharide standards have also been prepared using the same protocol. All the samples were analyzed by GC-MS in order to compare their spectra and retention times. GC-MS analyses were performed with a 6890N GC Interface with 5973 MSD by using an HP5-MS capillary column (25 m length 0.25 mm internal diameter and 0.25 μm internal diameter). The oven program was 70°C for 2 minutes, then a ramp to 300°C in 57 minutes, 10 minutes at 300°C. The scanning masses ranged from m/z 50 amu to m/z 650 amu. The on-column injected volume was 0.1 μL. The source temperature was 230°C, the quadrupole temperature set at 150°C, the He flow was 0.32 μL/min. Software was Chemstation.
Quantification of tumor cell number and binding
To evaluate tumor cell number, the pretreated or untreated macrophages were challenged with tumor cells for 72 hours. To evaluate the involvement of cell contact, macrophages were separated from EL4-luc2 cells by a transwell insert (0.4 µm pore size). For binding assays, macrophages were challenged with tumor cells for 2 hours at 4°C. Before challenge, tumor cells were treated or not with recombinant neumamidase A (Sigma). Non-fixed tumor cells were removed by washing. The luciferase activity was measured using t-luciferin (Caliper, 90 µg/mL). The binding of EL4 tumor cells and normal T lymphocytes with untreated or IL13-treated macrophages was also evaluated by confocal microscopy after CFSE labeling (Confocal ZEISS LSM780) of EL4 or T cells. ID8-luc cell death was evaluated with the LDH cytotoxicity assay Kit (Roche).

Metabolic assays, cytokine, 15-HETE, and mRNA quantification
IL13-treated or untreated macrophages were challenged with tumor cells for 48 hours. The arginase activity and nitrite release were measured as described previously (20). The arginase activity was evaluated in cell lysates after incubation with L-arginine (0.5 mol/L), and then the urea concentration was measured at 540 nm after addition of α-isonitrosopropiophenone. For nitrite release, Griess reagent was used to quantify the concentration of nitrite, which is a stable product of NO. The ROS production of macrophages was measured by chemiluminescence in the presence of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) using a thermostatically (37°C) controlled luminometer (Envision, PerkinElmer). The ROS production was monitored for 90 minutes (19). The cytokine releases of macrophages were evaluated by ELISA (BD Biosciences). The 15-HETE production of macrophages challenged with EL4-luc2 for 2 hours was measured by EIA (BD-HETE EIA kit, Cayman).

Real-time qPCR was performed on a LightCycler 480 system using LightCycler SYBR SYBR Green I Master (Roche Diagnostics). Actb (β-Actin) mRNA was used as the invariant control. Serially diluted samples of pooled cDNA were used as external standards in each run for the quantification. Primer sequences are listed in Supplementary Table S1.

Flow cytometry
For cell-cycle assay, after challenge with macrophages, EL4-luc2 cells were stained with propidium iodide (PI, BD Biosciences, 25 µg/mL). For cell death, EL4-luc2 cells were stained with Annexin V-FITC and propidium iodide (Annexin V: FITC Apoptosis Detection Kit I, BD Biosciences). For cell proliferation, EL4-luc2 cells were stained with CFSE (BD Biosciences, 10 µmol/L). IL13-treated or untreated macrophages were challenged with EL4-luc2 cells for 48 hours. Dectin-1, CD36, and MR were detected, respectively, using FITC-dectin-1 mAb (Serotec), PE-CD36 mAb (Santa Cruz), or FITC-mannosylated bovine serum albumin (Sigma; ref. 21).

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For immune cell infiltration and activation in ascites from untreated or IL13-treated EL4 tumor-bearing mice, peritoneal cells were collected for ELISA cytokine titration. The luciferase activity was measured on peritoneal cell samples and on each organ (spleen, lungs, and lymph nodes) with a luminometer (Envision, PerkinElmer). For in vivo Imaging System (IVIS), C57BL/6 mice were injected i.p. with EL4-luc2 cells (1×10^6 cells/mouse) or ID8-luc2 (5×10^6 cells/mouse) cells administered intraperitoneally (i.p.) in 0.2 mL saline solution. Liposomal Clodronate (500 µg/g mouse, i.p. injections were performed 1 day before tumor cell injection and then every 3 days. Control groups received saline solution. For Liposomal Clodronate (500 µg/g mouse, i.p. injections were performed 1 day before tumor cell injection and then every 3 days. Control groups received saline solution.

At 12 days after EL4-luc2 cells injection and 35 days after ID8-luc injection, mice were euthanized using CO2 asphyxia. Peritoneal cells were removed aseptically and the supernatants (ascite fluid) were collected for ELISA cytokine titration. The luciferase activity was measured on peritoneal cell samples and on each organ (spleen, lungs, and lymph nodes) with a luminometer (Envision, PerkinElmer). For in vivo Imaging System (IVIS), C57BL/6 mice were injected i.p. with EL4-luc2 cells (1×10^6 cells/mouse) or ID8-luc2 cells (1×10^6 cells/mouse) and treated or not i.p. with IL13 (n = 10 per group). At 3, 5, 8, and 12 days after EL4-luc2 cell injection, mice were anesthetized by isoflurane and administered with luciferin into the peritoneal cavity (150 µg/g body weight). Mice were imaged, 6 minutes after luciferin injection with 30 seconds exposure length, using the in vivo imaging system (IVIS, PerkinElmer). The luminescent images were analyzed using Living Image 4.4 software. For ID8-tumor-bearing mice, tumor progression was evaluated by quantification of tumor burden in abdomen using the in vivo imaging system, by weight gain and ascites volume.

ROS production, arginase activity, and real-time PCR were studied after macrophage adherence. To evaluate killing activity of macrophages, they were cocultured 72 hours with EL4-luc2 cells (luciferase activity).
Statistical analysis

All the results correspond to mean ± SEM of triplicates and are representative of at least three independent experiments. For each experiment, the data were subjected to one-way analysis of variance followed by the means multiple comparison method of Bonferroni–Dunnett using Statview software. For the survival study, statistical significance was determined by a log-rank test. \( P < 0.05 \) was considered statistically significant.

Results

IL13-activated macrophages promote EL4 tumor cell death by necrosis

Although IL6, a Th2 cytokine, significantly promoted protumor activity of macrophages, IL13- and IL4-activated macrophages inhibited EL4 tumor cell number similarly to Th1-driven macrophages (IFN\( \gamma \)/LPS- or TNF\( \alpha \)-stimulated macrophages; Fig. 1A).

Given that IL13 did not directly alter EL4 cell number, death, cycle, and index of proliferation (Supplementary Fig. S1A–D), we confirmed that IL13 antitumor activity is mediated through macrophages. Consistently, the genetic deletion of IL13 receptor subunit (Il4ra) in macrophages or of Stat6, a main factor involved in IL13 receptor-coupled signaling, totally abolished IL13-activated macrophages tumoricidal activity (Supplementary Fig. S1E).

After 24 hours of coculture with IL13-activated macrophages, EL4 cell number diminished over time (Fig. 1B), suggesting that IL13-activated macrophages exert a cytotoxic activity. Consistently, IL13-activated macrophages led to a significant arrest of EL4 proliferation following 48 hours of coculture (Fig. 1C). After 48 hours of coculture with IL13-activated macrophages, EL4 cell fraction in G0–G1, S, and G2–M phases decreased (Fig. 1D). This was accompanied by a corresponding increase in EL4 cell fraction in the sub-G0–G1 phase. The surviving cell population decreased over time, demonstrating that IL13-activated macrophages cause cytotoxicity in EL4 cells without cell-cycle arrest. As depicted in Fig. 1E, the increase of the percentage of cells stained with both Annexin V/PI and IP alone following 48 and 72 hours of

Figure 1.

IL13-activated macrophages induce EL4 tumor cell death by necrosis. A, Number of EL4-luc2 cells cultured in the presence (black bar) or not (white bar) of cytokine-treated macrophages. B–E, Number (B), proliferation assay (C), cell cycle (D), and cell death (E) of EL4-luc2 cells cultured in the presence of untreated or IL13-treated macrophages (M0). \(* P < 0.05\); \(* * P < 0.01\) compared with the respective untreated macrophages; \(* * * P < 0.001\) compared with the IFN\( \gamma \)-treated macrophages; and \# \( P < 0.05\); \# \( P < 0.01\) compared with the initial EL4-luc2 cell number (T0).
coculture with IL13-activated macrophages and the absence of increase of cells stained with Annexin V alone indicated that EL4 cell death is mediated by necrosis. Consistently, the use of Z-vad caspase inhibitor did not alter the reduced EL4 cell number induced by IL13-activated macrophages nor the percentage of EL4 cells stained with Annexin V/PI (Supplementary Fig. S1F and S1G). Necrostatin-1, a selective inhibitor of necroptosis, did not affect the reduced EL4 cell number induced by IL13-activated macrophages nor the percentage of EL4 cells stained with Annexin V/PI (Supplementary Fig. S1F and S1G). Necrostatin-1, a selective inhibitor of necroptosis, did not affect the reduced EL4 cell number induced by IL13-activated macrophages nor the percentage of EL4 cells stained with Annexin V/PI (Supplementary Fig. S1F and S1G). Necrostatin-1, a selective inhibitor of necroptosis, did not affect the reduced EL4 cell number induced by IL13-activated macrophages nor the percentage of EL4 cells stained with Annexin V/PI (Supplementary Fig. S1F and S1G).

Altogether, these results demonstrated that IL13-activated macrophage-induced cytotoxicity in EL4 cells is mediated by necrosis through the caspase-independent cell death pathway.

IL13-activated macrophage tumoricidal effects depend on ROS and arginase

The use of transwell insert culture dish (Fig. 2A) demonstrated that physical contact between IL13-activated macrophages and EL4 tumor cells is critical to generate soluble factor(s) contributing to the bystander tumoricidal effect of IL13-stimulated macrophages. EL4 cell number in the insert was decreased only when IL13-stimulated macrophages were simultaneously seeded with EL4 cells in companion plates (Fig. 2B). EL4 cell physical contact with IL13-activated macrophages robustly amplified the expression of standard IL13 activation markers such as Ym1 (Chi3l3), arginase-1 (Arg1), PPAR gamma (Pparg), Cd36, MR (Mrc1), and dectin-1 (Clec7a; Supplementary Fig. S2A and S2B). Concomitantly, EL4 cells also triggered Ncf1 (p47phox) expression, a cytosolic subunit of NADPH oxidase complex, phosphorylation of which is essential for ROS production and IL12, TNFα, and IL1β release (Supplementary Fig. S2A and S2C). Thus, EL4 tumor cells constituted a second signal to shift IL13-primed macrophages toward cytotoxic phenotype characterized by an induction of proinflammatory markers and a CLR signature composed of dectin-1 and MR.

The use of TNFα-deficient (Tnf−/−) macrophages showed that this cytokine was not involved in cytotoxic activity of

Figure 2.

ROS release and arginase are involved in the tumoricidal effect of IL13-activated macrophages. A, Schema of the transwell culture system. B, Number of EL4-luc2 cells in insert in the presence of untreated or IL13-treated macrophages cultured or not with EL4-luc2 cells in 24-well companion. C, Number of EL4-luc2 cells cultured with untreated or IL13-treated macrophages from Tnf−/− and Tnf+/+ mice. D–G, Nitrite release (D), ROS production (E), phosphorylated P47phox (F), and arginase activity (G) of untreated or IL13-treated macrophages in the presence or absence of EL4-luc2 cells. H and I, Number (H) and cell death (I) of EL4-luc2 cultured with macrophages stimulated or not with IL13, treated or not with NAC and/or L-arginine. **, P < 0.01 compared with respective untreated macrophages and *, P < 0.05; ***, P < 0.001 compared with IL13-treated macrophages without EL4 cells or to IL13-treated macrophages without NAC and L-arginine.
IL13-activated macrophages (Fig. 2C). Although NO secretion remained unchanged in IL13-activated macrophages stimulated by EL4 cells (Fig. 2D), these macrophages released a higher level of ROS (Fig. 2E). In line with this, phosphorylated p47phox was increased (Fig. 2F). Consistent with increased Arg1 mRNA expression in IL13-activated macrophages challenged by EL4 cells (Supplementary Fig. S2A), arginase activity was significantly higher (Fig. 2G). Finally, the use of the ROS scavenger NAC alone or the single supplementation of L-arginine in the medium partly inhibits the EL4 cell death induced by IL13-activated macrophages, demonstrating that their cytotoxic activity was dependent both on ROS and L-arginine amounts. The combination of NAC and of large amount of L-arginine in the medium completely abolished the tumoricidal response of IL13-stimulated macrophages, demonstrating that ROS release and the arginase activity-induced L-arginine deprivation are sufficient for their cytotoxicity (Fig. 2H and I).

Sialic acid binding through MR and dectin-1 is critical for tumoricidal activity

Because malignant transformation often correlates with altered glycosylation profile on tumor cell surface, we analyzed by GC/MS the sugar composition of normal lymphocytes and EL4 tumor cells (Fig. 3A; Supplementary Fig. S4A). We demonstrated a difference in the sugar composition between normal and abnormal T cells, due to the amino sugars and not to the usual hexoses and corresponding deoxyhexoses (Fig. 3A; Supplementary Fig. S4A). EL4 tumor cells exhibited higher amounts of sialic acid (NAc neuraminic acid) and NAc-Galactosamine than normal T cells.
IL13-activated macrophages had a greater ability to bind EL4 tumor cells (Fig. 3B). The pretreatment of EL4 tumor cells with neuraminidase, which cleaves the glycosidic linkage of sialic acid (Supplementary Fig. S4B), or the preincubation of IL13-activated macrophages with neuraminic acid, completely abolished their capacity to interact with EL4 tumor cells (Fig. 3B). Consistently, this binding was abrogated, in a dose-dependent manner, when IL13-activated macrophages were preincubated with sialylated LacNac, whereas it was not altered by the presence of nonsialylated LacNac (Fig. 3B; Supplementary Fig. S4C). We demonstrated that the normal T cells, which contain lower amounts of sialic acid at their surface, did not bind IL13-activated macrophages (Supplementary Fig. S4), highlighting sialic acid at the surface of EL4 cells as the critical epitope responsible for the physical interaction with IL13-activated macrophages.

The expression of siglec-1, well known for its specificity for sialic acid–containing glycans, and of CLRs, such as SIGNR-1 (Gil209b), SIGNR-3 (Gil209d), MGL (Clec10a), and Mincl (Clec4e), was not changed in IL13-activated macrophages in the presence of EL4 tumor cells (Fig. 3C). Only MR and dectin-1 mRNA expression was significantly increased (Fig. 3C). Consistently, deficiencies for MR (Mrc1+/−) or dectin-1 (Clec7a−/−) in IL13-activated macrophages resulted in a complete loss of their capacity to interact with EL4 cells (Fig. 3D), supporting the role of these CLRs in the physical interaction of IL13-activated macrophages with EL4 tumor cells.

Targeted deficiencies for MR or dectin-1 led to the complete loss of tumoricidal activity of IL13-activated macrophages (Fig. 3E and F). In line with previous data on ROS release and arginase activity in IL13-activated macrophages challenged with EL4 cells were affected by the absence of either Mrc1 or Clec7a (Fig. 3G and H). The robust induction of standard IL13 activation markers, Ncf1, and cytokine production in IL13-activated Mrc1+/+ or Clec7a+/+ macrophages was significantly diminished in Mrc1−/− or Clec7a−/− macrophages (Supplementary Fig. S5A–C).

We then determined whether the interaction between IL13-activated macrophages and EL4 cells occurs through the binding of tumoral sialic acid on MR/dectin-1 macrophage receptors (Fig. 3I). Similarity to EL4 cells, the sialic acid increased tumoral activity of IL13-activated Mrc1+/+ and Clec7a+/+ macrophages, as reflected by the decrease of EL4 cell number in insert, whereas this tumoral activity was lost in IL13-activated Mrc1−/− or Clec7a−/− macrophages (Fig. 3I). Altogether, these data reveal that the antitumor response of IL13-activated macrophages requires a physical contact depending on the recognition of tumoral sialic acid epitope by MR and dectin-1 receptors.

Cytotoxic function is induced by Syk/P47phox signaling and AA-HETE-PPARγ

We next dissected the signaling downstream of MR and dectin-1 involved in acquisition of cytotoxic functions by IL13-stimulated macrophages against EL4 tumor cells. The use of a selective Syk inhibitor Bay 61-3606 (Bay) revealed that IL13-activated macrophages cytotoxic activity was partially dependent on Syk (Fig. 4A). We showed that Bay abolished ROS production in response to EL4 cells (Fig. 4B) and did not affect arginase activity induction (Fig. 4C). The amount of phosphorylated p47phox in IL13-activated Mrc1+/− or Clec7a−/− macrophages was decreased (Fig. 4D). These data established a direct link between the Syk-coupled MR/dectin-1 signaling pathway, p47phox phosphorylation, and ROS release.

A direct link between MR/dectin-1 and arachidonic acid (AA) metabolism to trigger macrophage cytotoxic pathways was previously established (19). We demonstrated that in the presence of MAFP, an inhibitor of AA generation, IL13-activated macrophages antitumor activity was partly affected (Fig. 4A) and the induction of arginase was abolished (Fig. 4C). Although EL4 cell challenge resulted in the induction of AA release in IL13-activated Mrc1+/+ or Clec7a−/− macrophages, it failed to do so in IL13-activated Mrc1−/− or Clec7a−/− macrophages (Fig. 4E), demonstrating that AA-coupled MR/dectin-1 signaling is essential for the induction of arginase activity.

To explore the link between AA metabolism and arginase activity induction, we evaluated the implication of PPARγ, known to directly control arginase-1 expression (19), and to be activated by ligands derived from AA metabolism. The impaired induction of arginase activity in IL13-activated macrophages deficient for PPARγ (Pparg−/−; Fig. 4F) and the subsequent decrease of their cytotoxic activity (Fig. 4G) support the existence of a PPARγ-dependent mechanism in arginase activity amplification.

PPARγ is activated by endogenous ligands such as 15-deoxy-Δ12,14-PGJ2, metabolized through the cyclooxygenases and PGD synthase (Pgd2s), and the 12- and 15-hydroxyeicosatrienoic acids (HETE), metabolized through 12/15 lipoxygenases (Alox15; ref. 22). Although Pgd2s mRNA was not differentially expressed in IL13-activated macrophages upon EL4 challenge, Alox15 gene expression was significantly increased (Fig. 4H). Accordingly, 15-HETE production was enhanced in IL13-activated macrophages challenged by EL4 cells (Fig. 4I). This increased 15-HETE production was completely lost in IL13-activated Mrc1−/− or Clec7a−/− macrophages (Fig. 4I), indicating that EL4 tumor cell challenge with IL13-activated macrophages drives the generation of 15-HETE metabolites through the AA-coupled MR/Dectin-1 signaling pathway.

Finally, the addition of exogenous 15-HETE restored arginase activity defect (Fig. 4J) and decreased EL4 cell number (Fig. 4K) in IL13-activated Mrc1−/− or Clec7a−/− macrophages. In IL13-activated Pparg−/− macrophages, arginase activity and EL4 cell number was not affected by 15-HETE treatment (Fig. 4I and K), demonstrating that PPARγ is required for 15-HETE–mediated arginase and cytotoxic activities. Altogether, these results demonstrate that arginase activity induction in IL13-activated macrophages in response to EL4 tumor cells is dependent on the MR/dectin-1/AA-15-HETE/PPARγ axis.

In vivo IL13 treatment promotes antitumor properties of macrophages

We next showed that in vivo IL13 treatment significantly extended the survival of EL4 tumor–bearing mice (Fig. 5A). We ascribed the late mortality of IL13-treated mice to the decreased tumor burden in the abdomen, the lymph nodes, and spleen (Fig. 5B–D). Consistently, the ascites-dependent weight gain was significantly decreased (Fig. 5C), highlighting a potent role of IL13 in the inhibition of tumor development. Accordingly, compared with peritoneal macrophages from untreated EL4 tumor–bearing mice, macrophages from tumor ascites of IL13-treated mice showed an improvement in their ability to kill EL4 cells (Fig. 5E), to produce ROS (Fig. 5F) and presented a higher arginase activity (Fig. 5G).

The in vivo treatment with IL4, a Th2 cytokine that shares IL13 signaling, increased the macrophage ability to kill EL4 cells (Supplementary Fig. S6B), to produce ROS (Supplementary Fig. S6C), and to exert a higher arginase activity (Supplementary Fig. S6D).
In line with these results, IL4 treatment decreased ascites-tumor burden in IL4-treated EL4 tumor-bearing mice (Supplementary Fig. S6A). These data confirmed that IL4 and IL13 treatments exhibit similar effects in the orientation of macrophages toward a cytotoxic and tumoricidal phenotype and on lymphoma progression.

To further explore whether IL13 treatment affects TAMs phenotype, we first characterized peritoneal macrophages of EL4 tumor-bearing mice. As expected, EL4 tumor switches peritoneal macrophage phenotype toward tumor permissive status, characterized by an upregulation of M2 markers and an increase of immune tolerance factors such as *Il10*, *Il6*, and *Ido2* and of angiogenesis such as *Tgfb1* (Supplementary Fig. S6E). These macrophages promoted EL4 cell growth and were less efficient to produce ROS (Supplementary Fig. S6F and S6G). Upon IL13 treatment, macrophages from non-tumor-bearing mice presented an induction of standard IL13 activation markers, such as *Chi3l3*, *Arg1*, *Pparg*, *Cd36*, *Mrc1*, and *Clec7a*. This IL13-specific polarization signature was amplified in IL13-treated tumor-bearing mice (Supplementary Fig. S6H). Whereas the mRNA expression of *Tnf* and *Il1b* was decreased by IL13 treatment in macrophages from non-tumor-bearing mice, these genes were induced in macrophages from IL13-treated tumor-bearing mice. *Ncf1* expression was increased by IL13 treatment only in macrophages from tumor ascites. Except for *Il10*, of which expression is decreased by IL13 treatment only in macrophages from tumor-bearing mice, IL13 decreased the expression of immune tolerance and angiogenesis factors (*Il6*, *Tgfb1*, and *Ido2*) both in non-tumor and tumor-bearing mice (Supplementary Fig. S6H). Supporting these findings, we also observed that IL12, TNFa, and IL1b amounts in tumor ascites were elevated, whereas IL10 and IL6 concentrations were significantly reduced (Supplementary...
Fig. S6). Altogether, these data demonstrate that IL13 in vivo treatment is associated with a macrophage phenotype characterized by a decrease of immune tolerance and angiogenic factors, an induction of proinflammatory markers and a Dectin-1 and MR signature.

The capacity of IL13 treatment to orient immunosuppressive and protumor macrophages toward tumoridocic phenotype was reinforced by the capacity of in vitro IL13 treatment to reverse the protumor macrophages from EL4 tumor–bearing mice toward macrophages that inhibit EL4 cell growth (Supplementary Fig. S6I). To establish that IL13 antitumor activity is dependent on macrophages, we performed adoptive transfers of macrophages from IL13-treated mice into EL4 tumor–bearing mice. Mice receiving IL13-activated macrophages showed a significant decrease of the tumor burden in tumor ascites, lymph nodes, spleen, and lungs, as compared with respective untreated EL4 tumor–bearing mice (Fig. 5H and I). Consistently, macrophages from mice receiving IL13-activated macrophages showed an improvement in their ability to kill EL4 tumor cells (Fig. 5J) through an increase of their capacity to release ROS (Fig. 5K) and to exert arginase activity (Fig. 5L). The phenotypic characterization of transferred macrophages (F4/80+ MHC II+ Cell trace+) and host macrophages (F4/80+ MHC II+ Cell trace+) demonstrated that these two populations share the same pattern of gene expression (Supplementary Fig. S7A), suggesting a transfer of phenotype from transferred macrophages toward host macrophages.

The macrophage depletion by i.p. administration of clodronate in inhibited IL13-induced antitumor activities in tumor ascites, lymph nodes, spleen, and lungs (Supplementary Fig. S7B–D), reinforcing the involvement of macrophages in IL13 antitumor activity.

We demonstrated that clodronate treatment alone was less efficient in reducing tumor burden than macrophage conversion to an antitumor phenotype by IL13 treatment (Supplementary Fig. S7E), suggesting that the strategy to orient TAMs toward a cytotoxic phenotype to reduce tumor progression is more promising than their depletion. The decreased tumor burden in the peritoneum of IL13-treated mice was totally abolished in EL4 tumor–bearing Mrc1−/− or Clec7a−/− mice (Fig. 5M). In line with these results, the tumoricidal activity of macrophages from EL4 tumor–bearing Mrc1−/− or Clec7a−/− mice was completely lost (Fig. 5N). These results support in vivo the critical role of MR and dectin-1 recognition processes on macrophages in IL13-mediated antitumor activity. IL13 administration to EL4...
tumor-bearing mice did not affect the tumor adaptive immunity, because there were no detected changes in the percentage of CD4\(^+\) and CD8\(^+\) T cells, Tregs, B cells, and NK cells, or in the activation state of T cells (Supplementary Fig. S8).

IL13-activated macrophages have tumoricidal activity against ID8 cells

To investigate whether IL13-activated macrophage-induced cytotoxicity can be extended to another type of tumor, we evaluated the impact of IL13 treatment on murine ovarian adenocarcinoma cells (ID8). Although IL13 did not directly alter ID8 cell number, IL13-activated macrophages significantly inhibited their number over time (Fig. 6A and B). The increased LDH release by ID8 in the presence of IL13-activated macrophages suggested that the ID8 cell death is mediated by necrosis (Fig. 6C). Consistent with this finding, the use of Z-vad caspase inhibitor did not alter the reduced ID8 cell number induced by IL13-activated macrophages (Fig. 6D). IL13-activated macrophages released a greater amount of ROS (Fig. 6E) and exhibited significantly higher arginase activity after challenge with ID8 (Fig. 6F), suggesting that ROS and L-arginine are involved in their cytotoxic activity against ID8 cells.

ID8 tumor cells, which express great amounts of sialic acid (NeuNac) at their surface (Fig. 6G), interacted with IL13-activated macrophages through sialic acid because the pretreatment of ID8 tumor cells with neuraminidase completely abolished their capacity to bind with ID8 (Fig. 6H). IL13-activated macrophages deleted for MR or dectin-1 failed to bind ID8 cells (Fig. 6I). These results demonstrate that the binding of sialic acid present at the surface of ID8 tumor cells through MR and dectin-1 is essential in the tumoricidal activity of IL13-activated macrophages.

IL13 promotes tumoricidal activity of macrophages against ovarian adenocarcinoma cells (ID8). A, ID8-luc cell number cultured in the presence or absence of IL13-activated macrophages. B, ID8-luc cell number cultured with untreated or IL13-treated macrophages during 24, 48, or 72 hours. C, ID8-luc cell death in the presence of untreated or IL13-treated macrophages. D, Number of ID8-luc cells in the presence of macrophages stimulated or not with IL13, treated or not with Z-vad. E–F, ROS production (E) and arginase activity (F) of untreated or IL13-treated macrophages in the presence or absence of ID8-luc cells. G, Chromatogram specific for acetylated and methylated amino sugars at the surface of ID8-luc cells. Numbers are intensities × 10\(^6\). H, Binding of ID8-luc cells, pretreated or not by neuraminidase (NA), on untreated or IL13-treated macrophages. I, Binding of ID8-luc cells on untreated or IL13-treated Mrc1\(^{-/-}\) or Clec7a\(^{-/-}\) macrophages.

In vivo IL13 treatment of ovarian adenocarcinoma-bearing mice decreased tumor burden in the abdomen and ascites-dependent weight gain, highlighting a potent role of IL13 in the inhibition of ovarian adenocarcinoma growth (Fig. 6J). Altogether, these data support the extension of cytotoxic activity of IL13-activated macrophages to various tumor cells.

IL13 promotes tumoricidal activity in human MDMs

We evaluated the impact of human MDMs activated by IL13 on human T-cell leukemia cells (Jurkat cell line). Although the exposure of Jurkat tumor cells with IL13 did not directly alter their number (Fig. 7A), IL13-activated human MDMs significantly decreased Jurkat tumor cell number (Fig. 7B). The use of NAC and the L-arginine medium supplementation demonstrated that the cytotoxic effect of IL13-activated human MDMs is completely dependent on ROS production and arginase activity (Fig. 7B). Consistently, IL13-activated human MDMs released large amounts of ROS in response to Jurkat tumor cells (Fig. 7C), and their arginase activity was also significantly increased (Fig. 7D).
Jurkat tumor cells contain high amounts of sialic acid at their surface (Fig. 7E), and the pretreatment with neuraminidase completely abolished their capacity to bind with IL13-activated macrophages (Fig. 7F). IL13-activated human MDMs lost their tumoricidal activity against Jurkat tumor cells after silencing of MR (Mrc1) or dectin-1 (Clec7a; Fig. 7G), underscoring the importance of these CLRs on IL13-activated macrophages in the recognition of human tumor cells and hence in the acquisition of antitumor phenotype.

To investigate whether IL13-activated human MDM-induced cytotoxicity can extend on other tumor types, we evaluated the impact of IL13 treatment on human breast adenocarcinoma (MDA-MB-231) cells. Similarly to Jurkat cells, the number of MDA-MB-231 cells was significantly decreased by IL13-activated human MDMs (Fig. 7H), which ROS amounts were induced by 2-fold (Fig. 7I), and exhibited higher arginase activity in response to MDA-MB-231 cells (Fig. 7J). These tumor cells interacted with IL13-activated macrophages through sialic acid (NeuNac; Fig. 7K) and expressed great amounts of this glycan epitope at their surface (Fig. 7L). Inversely, the data on another tumor cell line, the human ovarian carcinoma cells (Skov-3), revealed that antitumor activity of IL13-activated human MDMs is concealed by the direct pro-tumor effect of IL13 on these tumor cells (Fig. 7M). This direct protumor activity of IL13 on Skov-3 tumor cells is accompanied by surface expression of IL13RA2 (IL13RA2; Fig. 7N), a receptor known to promote cancer invasion and proliferation (23, 24).
absence of the direct protumour activity of IL13 on Jurkat and MDA-MB-231 tumour cells is reinforced by the lack of IL13Ra2 expression on their surfaces (Fig. 7N). Altogether, these results establish that the macrophage antitumor effect mediated via the recognition of tumor sialic acid epitope by MR and dectin-1 can extend to various types of human tumors. They also highlight the restrictive use of IL13 to induce these CLRs due to the expression of IL13Ra2 on some tumor cells.

Discussion

Because TAMs are subject to local levels of many factors that lead to protumorigenic macrophages, their education within the tumor toward tumoricidal macrophages appears to be a potential strategy for cancer therapy. Several lines of evidence suggest that the acquisition of cytotoxic functions in macrophages depends on the expression and activation of CLRs (16, 25), which are highly expressed in the Th2 microenvironment. In this context, we determined how IL13 modulates tumoricidal properties of macrophages during T-cell lymphoma and ovarian adenocarcinoma development. We demonstrated here that IL13 treatment inhibits T-cell lymphoma and ovarian adenocarcinoma development in tumor-bearing mice through the activation of antitumor properties of macrophages. This is consistent with a decrease of the tumor burden in tumor ascites, lymph nodes, spleen, and lungs after adoptive transfer of IL13-activated macrophages in EL4-bearing mice. Our results provide mechanistic insight into the antitumor effect of IL13 and corroborate previous findings showing that, associated with the expression of typical markers of B16F1 melanoma and MethA fibrosarcoma cells (26, 27), Local administration of IL13 at the site of transplanted tumor cells in vivo had potent inhibitory effects on tumor growth, probably resulting from pleiotropic effects, such as the recruitment of non-specific cells like monocytes, macrophages, and neutrophils into the tumor (26, 27). The advantageous effect of IL13 was supported by an inverted correlation between the serum level of IL13 and factors reflecting tumor progression (28).

The antitumor effect of IL13 is associated with a macrophage phenotype characterized by a significant decrease of immune tolerance and angiogenic factors. Results also demonstrate an induction of IL13 standard markers, in particular Dectin-1, MR, and Arginase-1, associated with the expression of typical markers of IL13 activation. Thus, the tumor microenvironment can induce changes in gene expression with the appearance of atypical markers in macrophages, which phenotype has already been oriented by IL13 treatment.

Despite the growing knowledge of the role of CLRs in pathogen elimination, little is known about their contribution to antitumor responses. Here, we demonstrated that the tumoricidal properties of macrophages from IL13-treated EL4-bearing mice were abolished in mice deleted for MR or dectin-1, supporting the critical role of these receptors in IL13-mediated antitumor activity. IL13-activated macrophages are able to shift toward a tumoricidal state upon the “triggering” signal provided by tumor cell recognition through MR and dectin-1. We identified sialic acid as a critical epitope at the surface of tumor cells responsible for their interaction with MR and dectin-1, highlighting the glycan specificity of the cooperation between dectin-1 and MR for sialic acid. These data support that heterodimeric complex formation among different CLRs diversifies host PRR repertoire and hence expand the ligand spectrum (29, 30).

The binding of tumor sialic acid through MR and dectin-1 is essential in the tumoricidal activity of IL13-activated macrophages against tumor cells. Indeed, dectin-1 at the surface of macrophages is critical to recognize tumor cells that highly express glycan structures and to activate NK-mediated tumor cell killing (16). In line with this, the expression on tumor cells of ligands for the NKG2D receptor, a CLR expressed by NK cells, can also augment T-cell responses, demonstrating that this CLR mediates beneficial immune responses against tumors (15). Here, the specific contribution of dectin-1 and MR is reinforced by a lack of induction of other macrophage lectin-like receptors, such as DC-SIGN, MGL, Mincle, and Siglec-1, known to be involved in immune escape (18). The identification of MR and dectin-1 as extracellular sensors for tumoricidal function promotion represents a major breakthrough in the host–tumor cell interaction and host-mediated tumor cell elimination. Altogether, these data support that the CLRs on macrophages may be an adequate system to orchestrate an antitumor innate immune response for early detection and removal of tumor cells with aberrant glycosylation, similar to their role in microbial glycan recognition and pathogen elimination.

The investigation of the signaling downstream of MR and dectin-1 identified ROS and arginase as factors involved in the cytotoxic effect of IL13-activated macrophages against tumor cells. Consistent with the involvement of MR and dectin-1 in the induction of ROS production during infections through a signaling cascade requiring Syk (18, 19, 31), we demonstrated, in the context of the tumor, a direct link between the Syk-coupled MR/dectin-1 signaling pathway, p47phox phosphorylation, and ROS release. We showed that the induction of arginase activity requires MR/dectin-1–coupled AA mobilization, 15-HETE generation, and the subsequent PPARγ activation. This was supported by the fact that the increase of CLRs favors arginase activity in response to parasites (25, 32, 33) and that MR/Dectin-1 triggers the AA pathway during Leishmania infection (19).

This study also validated that IL13-activated human macrophages display similar contributions against human T-cell leukemia cells. We revealed that IL13 antitumor effectiveness in human macrophages can extend to other tumor types as demonstrated in a human breast adenocarcinoma cell line. However, we also evidenced that the gene-expression signature of IL13Ra2 receptor on tumor cells, a receptor known to promote cancer invasion and proliferation (23, 24), is critical and determines the use and the efficacy of IL13.

The positive or negative roles of IL4 and IL13 cytokines in tumor immunity are closely associated with their sources. Although endogenous IL4 and IL13 were reported to promote tumor growth, exogenous IL4 and IL13 delivered as recombinant protein into the host often suppress tumor development (34, 35). Several studies of Th2 cytokine neutralization, using IL4 or IL13 knockout mice or IL4 or IL13-specific antibodies, demonstrated a decrease of tumor growth and metastasis related to an enhanced Th1 response (34, 35). Coussens and colleagues demonstrated that the significant diminution of mammary adenocarcinoma metastasis in mice models of immune depletion of CD4+ T cells or of IL4 was mediated by a suppression of protumor properties of TAMs (36). However, several experiments using the inoculation of exogenous IL4 or IL13 showed these cytokines to be potent antitumor agents, as they induce tumor rejection in different types of tumors (26, 34, 35). These tumor-inhibiting effects of IL4 and IL13 were associated with the maturation of myeloid...
precursor cells toward inflammatory effectors and were also dependent on tumor-infiltrating eosinophils (37). In addition, IL4-activated tumor-infiltrating dendritic cells were described to promote tumor-specific cytotoxic T-cell responses (34, 35). Consistent with a Th2-driven antitumor effect through the stimulation of innate immune system, we demonstrated in this study that exogenous IL13 treatment decreased tumor burden by enhancing the antitumor properties of TAMs. Altogether, these results established that both Th1 and Th2 responses can be involved in antitumor immunity, suggesting that the most effective cancer immunotherapies may be those that can simultaneously associate multiple Th1 and Th2 effector mechanisms that can cooperate for maximal systemic antitumor response (38).

Although the inhibition of IL13 has received a great deal of attention as a new therapeutic strategy for tumor diseases, IL13 can promote tumoricidal activities of TAMs and inhibit cancer cell proliferation (5–7, 41), supporting that this cytokine plays a complex role in tumor development according to the type of malignancy, and therefore its therapeutic control is critical and should be considered with caution. In this context, our findings revealing that the antitumor effects are similar when we administrate IL13 directly or IL13-polarized macrophages offer a new strategy to avoid the possible deleterious effects of the direct administration of IL13. Our results showing that IL13 and IL4 treatments exhibit similar effects in the orientation of macrophages toward a cytotoxic and tumoricidal phenotype and on tumor cell proliferation (39–41), supporting that this cytokine plays a complex role in tumor development according to the type of malignancy, and therefore its therapeutic control is critical and should be considered with caution. In this context, our findings revealing that the antitumor effects are similar when we administrate IL13 directly or IL13-polarized macrophages offer a new strategy to avoid the possible deleterious effects of the direct administration of IL13.

In conclusion, we have shown that IL13 exhibits efficient activity against T-cell lymphoma and ovarian adenocarcinoma through the enhancement of macrophage antitumoral properties. Our findings suggest that IL13 strengthens tumor immunosurveillance by increasing expression of CLRs, enabling early detection and removal of tumor cells with aberrant glycosylation.

Finally, these results identify MR and dectin-1 as main components of macrophage antitumoral response and offer attractive targets to promote TAMs' tumoricidal properties.

Disclosure of Potential Conflicts of Interest

P. Balard reports receiving a commercial research grant from Nutergia. No potential conflicts of interest were disclosed by the other authors.

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References

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