Basophil Recruitment into Tumor-Draining Lymph Nodes Correlates with Th2 Inflammation and Reduced Survival in Pancreatic Cancer Patients

Lucia De Monte1,2, Sonja Wörmann3, Emanuela Brunetto1,2,4, Silvia heltai1,2, Gilda Magliacane5,6, Michele Reni6,7, Anna Maria Paganoni8, Helios Recalde9, Anna Mondino2,10, Massimo Falconi4,6,11, Francesca Aleotti6,11, Gianpaolo Balzano6,11, Hana Algül8, Claudio Doglioni4,5,6, and Maria Pia Protti1,2

Abstract

In pancreatic ductal adenocarcinomas (PDAC), lymphoid infiltrates, comprised mainly of Th2 cells, predict a poor survival outcome in patients. IL4 signaling has been suggested to stabilize the Th2 phenotype in this setting, but the cellular source of IL4 in PDAC is unclear. Here, we show that basophils expressing IL4 are enriched in tumor-draining lymph nodes (TDLN) of PDAC patients. Basophils present in TDLNs correlated significantly with the Th2/Th1 cell ratio in tumors, where they served as an independent prognostic biomarker of patient survival after surgery. Investigations in mouse models of pancreatic cancer confirmed a functional role for basophils during tumor progression. The recruitment of basophils into TDLN relied partly upon the release of chemokine CCL7/MCP3 by "alternatively activated" monocytes, whereas basophil activation was induced by T-cell–derived IL3. Our results show how basophils recruited and activated in TDLNs under the influence of the tumor microenvironment regulate tumor-promoting Th2 inflammation in PDAC, helping in illuminating a key element of the immune milieu of pancreatic cancer. Cancer Res; 76(7); 1792–803. © 2016 AACR.

Introduction

PDAC is a very aggressive disease with dismal prognosis (1). The cross-talk within the tumor microenvironment among different cellular components has a negative implication for disease prognosis (2–8).

We previously reported that in PDAC, the ratio of GATA-3+ (Th2) over T-bet+ (Th1) stromal lymphoid infiltrates is an independent predictive factor of the survival of patients after surgery (9). We identified the thymic stromal lymphopoietin (TSLP), which is produced by activated cancer–associated fibroblasts (CAF), as a key cytokine for conditioning myeloid dendritic cells (DC) with Th2-polarizing capability that reside within the tumor stroma and in tumor-draining lymph nodes (TDLN refs. 9, 10).

How Th2 immune responses are initiated and amplified in vivo and, specifically, which are the relevant accessory cells and cytokines involved is still highly debated and possibly depends on the model (11). Although Th2 cell differentiation may occur in the absence of IL4 (12), the IL4/STAT6 pathway has a main role in the induction of GATA-3 expression in T cells for the Th2 phenotype stabilization (13). DCs have been reported to prime Th2 responses in several models under the influence of Th2-polarizing cytokines, comprising TSLP (14, 15); however, DC incapacity to produce IL4 has prompted the search for identifying accessory cells that provide in vivo the early innate source of IL4.

Proposed sources of IL4 had included eosinophils, mast cells, basophils, natural killer T cells, and CD4+ T cells (16). Recently, basophils have been shown in different mouse models of helminth infections to contribute to Th2 cell development by secreting IL4 after their transient recruitment into draining lymph nodes, where DCs are the antigen-presenting cells primarily responsible for Th2 cell priming (17, 18).

We hypothesized that in PDAC, basophils might be the source of IL4 necessary to induce GATA-3 expression in Th2 cells induced by TSLP-conditioned DCs in TDLNs, thus functioning as accessory cells during full development/maintenance of Th2 immune responses.

Here, we evaluated first the presence of basophils in TDLNs and their prognostic significance in PDAC patients; second, we supported the role of basophils in tumor development/progression using basophil-deficient mice; and third, we performed ex vivo and in vitro experiments to address the mechanisms of basophil recruitment and activation.
Patients and Methods

Patient samples and patient population enrolled for survival analyses

A total of 85 PDAC patients were prospectively or retrospectively evaluated in the study, and tumor and lymph node specimens were collected at surgery. TDLNs were collected from fresh surgical specimens and non-TDLNs from the distal mesentry. Patients were classified in stage IA (1 patient), stage IIA (10 patients), stage IB (2 patients), stage IIB (71 patients), and stage III (1 patient) according to the 2002 staging criteria of AJCC (19).

The clinical characteristics of the 36 stage IB-III patients considered for survival analyses are reported in Table 1. Retrospective evaluation was also performed in TDLNs from patients suffering from intraductal papillary mucinous neoplasm (n = 4), solid pseudopapillary neoplasm (n = 4), and neuroendocrine tumor (n = 5). The Institutional Ethics Committee (Comitato Etico Fondazione Centro San Raffaele, Istituto Scientifico Ospedale San Raffaele, Milan, Italy) approved the study protocol and written consent was obtained from all donors.

Establishment of CAF cell lines from tumor specimens and isolation of basophils, T cells, and monocytes from lymph nodes

CAFs obtained by the outgrowth from tumor specimens were characterized and activated for TSLP secretion as described in ref. 9. Samples of TDLN and non-TDLNs were either immediately frozen in RNAlater (Ambion) for RNA extraction or freshly processed to obtain single-cell suspension for the isolation of basophils, T cells, or monocytes/macrophages. Basophils were isolated by negative selection with Basophil Isolation Kit II (Miltenyi Biotec) for phenotype analysis. T cells were isolated by negative selection with Basophil Isolation Kit II (Miltenyi Biotec), whereas CD14 MicroBeads (Miltenyi Biotec) were used to positively select monocytes/macrophages.

Mice and mice-derived tumor cell lines

Mep8Cre mice (20) were kindly provided by Prof. David Voehringer (University of Erlangen-Nuremberg, Erlangen, Germany). LSL-KrasG12D knockin (21), Ptf1a-creERT2 (22), and p53flfl mice were interbred to obtain compound mutant LSL-KrasG12D, p53flfl, Ptf1a-creERT2 termed KPc mice. LSL-KrasG12D knockin and Ptf1a-creERT2 were interbred to obtain compound mutant LSL-KrasG12D, Ptf1a-creERT2 termed Kras mice and used at 9 weeks of age, when the malignant lesions are not developed yet, as negative controls. C57BL/6 mice were used as wild type (WT). All procedures were conformed to the regulatory standards and approved by the Regierung von Oberbayern. Primary tumor cell lines were established from KPc pancreatic tumors and cultured until passage 6 to 8 in DMEM 10% FBS.

Real-time PCR

Total RNA was extracted from surgical specimens, purified cell subsets, and murine pancreatic samples using RiboPure Kit (Ambion) or RNeasy Mini Kit (Qiagen) according to the manufacturers’ instructions. A total of 50 to 100 ng cDNA/sample obtained with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for qRT-PCR. Murine cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). TaqMan primers specific for human IL4, IL3, CCL7/MCP3, CCL17/TARC, CCL11/eotaxin-1, CCL24/eotaxin-2, and CCL26/eotaxin-3 and murine Gata-3, T-bet, and Tslp (Applied Biosystems) were used. qRT-PCR analysis was performed as described previously (9, 22). Data were normalized to HGPRT or β-actin levels for human- and to endogenous cyclophilin for mice mRNA, respectively. Fold induction was calculated by the 2−ΔΔCt method.

In situ hybridization

The labeling of complementary RNA strands to localize specific IL4 expression in lymph node from PDAC patients and T-bet and GATA-3 RNA sequences in pancreatic tumors from KPc mice was performed applying the RNAscope 2.0 FPPE Assay (Advanced Cell Diagnostics), according to the manufacturer’s protocol. Microscopic observation was performed using Nikon Eclipse 80i and images digitalized using Aperio ScanScope (Nikon Instruments S.p.A.).

Orthotopic transplantation model

Cells (1 x 10⁶) from primary tumor cell lines established from KPc mice were orthotypically transplanted into 8-week-old Mep8Cre and WT mice. Mice were sacrificed at the indicated time points.

IHC

IHC was performed on tissue sections from tumor and lymph node specimens as detailed in ref. 24. The following antibodies were used: anti-pro-MBP1 (BioLegend, clone 11757-7D4), anti-T-bet (Santa Cruz Biotechnology, Inc.), anti-GATA-3 (R&D Systems), anti-CD11c (Novocastra, NCL-CD11c-536), and anti-CD14 (Novocastra). The quantification of GATA-3+ and T-bet+ lymphoid cells was performed as described in ref. 9. For pro-MBP1, up to 4 representative areas per sample within the lymph node medulla were selected; pro-MBP1+ cells were quantified with the Aperio ImageScope Nuclear Algorithm, and the average percentage of the pro-MBP1+ positive cells of the analyzed regions was reported. Sections used for IL4 in situ hybridization were stained with anti-pro-MBP1 antibody utilizing an alkaline phosphatase–based polymer system and a New Fuchsin chromogen (Bond Polymer Refine Red). Double immunostaining was performed visualizing first CD11c or CD14 with DAB or New Fuchsin, followed by pro-MBP1 reaction developed in red with New Fuchsin or in brown with DAB, respectively. Murine lymph

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Table 1. Characteristics of the 36 stage IB-III patients grouped by the median percentage of pro-MBP1-positive cells in TDLNs

<table>
<thead>
<tr>
<th>Variable</th>
<th>% pro-MBP1 &lt; 0.44 (n = 18)</th>
<th>% pro-MBP1 ≥ 0.44 (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender: female</td>
<td>11 (61%)</td>
<td>10 (56%)</td>
</tr>
<tr>
<td>Median age</td>
<td>67</td>
<td>62.5</td>
</tr>
<tr>
<td>Tumor site: head</td>
<td>14 (78%)</td>
<td>18 (100%)</td>
</tr>
<tr>
<td>Tumor size &gt;3 cm</td>
<td>3 (17%)</td>
<td>5 (28%)</td>
</tr>
<tr>
<td>Stage IIB/III</td>
<td>16 (89%)</td>
<td>13 (72%)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>6 (34%)</td>
<td>5 (28%)</td>
</tr>
<tr>
<td>Karnofsky ≥ 80</td>
<td>12 (67%)</td>
<td>12 (67%)</td>
</tr>
<tr>
<td>Surgical margin R1</td>
<td>5 (28%)</td>
<td>10 (56%)</td>
</tr>
<tr>
<td>Treatment: PEFGa</td>
<td>8 (45%)</td>
<td>11 (61%)</td>
</tr>
</tbody>
</table>

*Patients who had R0 or R1 resection of a stage IB-III PDAC, ages 18–75 years, and Karnofsky performance status ≥60 were eligible for adjuvant therapy. The patients were required to have postoperative treatment initiation within two months from surgery, no previous chemotherapy or radiotherapy for PDAC, adequate bone marrow, liver, and kidney. After tumor resection, 17 patients were treated with gemcitabine (48), and 19 patients received the PFEG regimen consisting of cisplatin, epirubicin, gemcitabine, and 5-fluorouracil (50). In both cases, chemotherapy was administered for 3 months, followed by chemoradiation (50).
nodes were stained with mMCP-8 (TUG8 clone) rat anti-mouse antibody (BioLegend) utilizing an anti-rat DAB polymer detection system (Rat-on-Mouse polymer, Biocare Medical). Staining quantification was performed as above.

**Basophil and monocyte isolation from human blood**

Basophils and monocytes were purified from blood buffy coats from healthy donors (HD). Basophil enrichment was performed as described in ref. 25. The basophil-enriched fraction was further purified with Basophil Isolation Kit II (Miltenyi Biotec). Monocytes were selected with CD14 MicroBeads (Miltenyi Biotec) from Ficoll gradient–isolated peripheral blood mononuclear cells.

**FACS analysis**

Basophils were stained with CD123-PE (BD Biosciences), CD203c-APC (Miltenyi Biotec), and FcRRII-APC, c-kit-PE, or c-kit-BV421 (BD Biosciences)–specific antibodies. Naïve CD4⁺ T cells were stained with CD4 and CD45RA–specific antibodies (BD Biosciences). CD3-APC, CD4-PE, and CD8-FITC antibodies (BD Biosciences) were used to quantify T cells recovered from the lymph nodes. Sample acquisition and analysis were performed with FACS Canto instrument and FlowJo software (LLC), respectively.

**Myeloid DC isolation and activation**

A total of 97% to 99% pure myeloid DCs were obtained as described in ref. 9 and cultured for 24 hours at 1 × 10⁶/mL in 96-well plates in Iscove’s Modified Dulbecco’s Medium (IMDM) 2% FBS ± the following stimuli: 25 ng/mL TSLP (R&D Systems). Day 4 was identified as the optimal condition and used for the basophil activation experiments.

**T-cell–derived IL3-dependent basophil activation**

Basophils (1 × 10⁶/mL) were plated in 24-well plates and cultured for 24 hours in IMDM 10% FBS ± the following stimulants: 5 ng/mL IL3 (R&D Systems) and supernatants from T cells cocultured with DCs (containing 60–200 pg/mL IL3). IL3-neutralizing or isotype control antibodies (R&D Systems) were added at 1 pg/mL. Basophil activation was measured by FACS analysis by quantitating CD203c expression, as reported in refs. 26, 27.

**Basophil migration assay**

CD14⁺ monocytes (1 × 10⁶/mL) were plated in 24-well plates in IMDM 5% FBS ± untreated or Act CAF supernatants. After 4 days, IL4-secreting basophils are significantly increased in TDLNs compared with non-TDLNs of PDAC patients. **A, IL4 mRNA expression in paired TDLNs and non-TDLNs (n = 20).** Significance was determined using one-tailed Wilcoxon matched pairs signed rank test (P = 0.015). **B, representative (n = 3) flow cytometry analysis for basophil (CD123⁺/FcrRII⁺/c-kit⁻) in TDLNs and non-TDLNs of patient #54.** **C, side scatter.** Representative (n = 18) immunohistochemical analysis for basophil (pro-MBP1) in TDLNs and non-TDLNs of patient #5. **D, arrows, pro-MBP1-positive cells.** **E, quantification of the percentage of basophils in the 18 paired samples tested.** Significance was determined using one-tailed Wilcoxon matched pairs signed rank test (P = 0.0009). **F, quantification of the percentage of basophils in TDLNs from PDAC and pancreatic benign tumors, IPMNs (n = 4), pseudopapillary tumors (n = 5), and neuroendocrine tumors (n = 5).** Significance was determined using one-tailed Mann–Whitney test and P = 0.002, P = 0.007, and P = 0.005, respectively. **G, IL4 mRNA expression in basophil-enriched and nonenriched cell populations (n = 5).** Significance was determined using one-tailed Wilcoxon matched pairs signed rank test (P = 0.0313). **H, plot of the percentage of basophils and IL4 mRNA expression in TDLNs from the same patients (n = 11).** Significance of the correlation was determined using nonparametric two-tailed Spearman r test (r = 0.6727, P = 0.0277). IsoAb, isotype antibody.
hours at 37°C, the cells were washed and medium replaced for 24 hours. Supernatants were collected, CCL7/MCP3 quantified by ELISA (DuoSet R&D Systems), and stored at −20°C for further use. Basophil migration was performed as described in ref. 28, with minor modifications. Briefly, 2 × 10⁶ basophils were added to the upper chamber and monocyte supernatants placed in the lower chamber. Anti-CCL7/MCP3-neutralizing or isotype control antibodies (R&D Systems) were added to the monocyte supernatants at 5 μg/mL. After 2.5 hours at 37°C incubation, migrated cells were collected and stained with APC-conjugated mouse anti-human FcεRI and PE-conjugated mouse anti-human CD123 antibodies (Miltenyi Biotec). Each sample was resuspended in a constant volume and acquired for 3 minutes with FACSCanto and analyzed with FlowJo software. Migration was expressed as the absolute number of basophils (FcεRI⁺CD123⁺) counted in the well. Similar experiments were performed with anti-CCL24/eotaxin-2-neutralizing antibody (R&D Systems).

Statistical analysis
To test the stochastic order between nonnormal distributions of paired data, one-tailed Wilcoxon matched pairs signed rank test was used. In case of independent distributions, one-tailed Mann–Whitney test and one-tailed Student t test for normal data were applied. Correlation between variables was determined by means of nonparametric two-tailed Spearman r test. Pearson χ² test was used to compare proportions. The survival curves were estimated with univariate analyses according to the Kaplan–Meier method and compared using the log-rank (Mantel–Cox) test. Multivariate analyses by Cox proportional hazard regression models were performed to estimate the independent potential risk factors that influence disease-free survival (DFS) and overall survival (OS). Values of \( P < 0.05 \) were considered significant. Analyses were carried out using the Statistica 4.0 statistical package for Windows (StatSoft) and R (R version 3.0.0).

Figure 3.
Basophil-deficient mice are resistant to full tumor development. A, in situ hybridization for mRNA expression of T-bet and Gata-3 in tumor stroma from KPC mice (\( n = 2 \)). B and C, ratio of the Gata-3/T-bet (B) and Tslp (C) mRNA expression in pancreata from KPC, Kras at 9 weeks of age, and WT mice. Significance was determined by two-tailed Mann–Whitney test. Gata-3/T-bet, KPC versus Kras (\( P = 0.017 \)) and KPC versus WT (\( P = 0.035 \)), TSLP, KPC versus WT (\( P = 0.022 \)), Kras versus WT (\( P = 0.032 \)). D, representative staining for MCP-8 (murine basophil marker) in tumor-associated lymph nodes in tumor-bearing KPC mice (\( n = 8 \)) and peripancreatic lymph nodes in Kras mice at 9 weeks of age (\( n = 4 \)). E, quantification of the percentage of basophils from D. Significance was determined by one-tailed Mann–Whitney test (\( P = 0.004 \)). F, representative hematoxylin and eosin (H&E) staining in WT (\( n = 5 \)) and Mcpt8Cre (\( n = 4 \)) mice at 1 week after orthotopic injection of tumor cells from KPC mice. G, representative hematoxylin and eosin staining in WT (\( n = 4 \)) and Mcpt8Cre (\( n = 4 \)) mice at 8 weeks after orthotopic injection of tumor cells from KPC mice. H, MCP-8 staining for basophils detection in tumor-associated lymph nodes in WT mice and in peripancreatic lymph nodes in Mcpt8Cre mice. I, quantification of the percentage of basophils from H. Significance was determined by one-tailed Mann–Whitney test (\( P = 0.009 \)). J, percentage of tumor-bearing mice after 1 and 8 weeks after implant. Significance at 8 weeks between the two groups (WT vs. Mcpt8Cre) was calculated with Pearson χ² test (\( P = 0.0012 \)).
Basophils in Pancreatic Cancer

Results

**IL4-expressing basophils are significantly increased in TDLNs compared with non-TDLNs in human PDAC.**

We previously reported that myeloid DCs conditioned by activated CAF–derived TSLP are sufficient to prime in vitro differentiation of naïve CD4+ T cells toward Th2 (9). However, as IL4 exerts a key role to stabilize the Th2 phenotype (11, 13), we looked for its expression in PDAC surgical samples and TDLNs. Although IL4 was not usually present in tumor samples (data not shown), it was consistently detectable in TDLNs and significantly increased with respect to non-TDLNs (Fig. 1A), suggesting that IL4-secreting cells were recruited to TDLNs where Th2 cell priming occurs. We focused on basophils, which have been recently recognized to exert non-redundant roles in Th2-protective immunity against parasites and in allergy through IL4 secretion (17, 18). To assess their presence in TDLNs, we studied a cohort of 47 stage IB-III patients. The percentage of basophils in TDLNs signifi- cantly correlates with predominant Th2 inflammation and predicts survival after surgery in PDAC patients.

To determine the possible correlation between predominant Th2 cell infiltration in the tumor and the recruitment of basophils into TDLNs, we studied a cohort of 47 stage IB-III patients. The cohort was composed by patients belonging to the previous cohort was composed by patients belonging to the previous
Figure 5.
CAF-conditioned monocytes induce basophil recruitment into TDLNs that is partially dependent on CCL7/MCP3. A, CCL7/MCP3 mRNA expression in paired TDLNs and non-TDLNs (n = 5). Significance was determined using one-tailed Wilcoxon matched pairs signed rank test (P = 0.031). B, CD14⁺ cells were purified from the blood of HDs and activated in vitro with the indicated stimuli for 5 hours, and tested for CCL7/MCP3 and CCL17/TARC mRNA expression (n = 2). Medium alone (med) and lipopolysaccharide (LPS) were used as controls. (Continued on the following page.)
cohort analyzed in ref. 9 and by newly prospectively recruited patients, on which we performed the analysis of the GATA-3 and T-bet expression in the tumor. We found a statistically significant correlation between the percentage of basophils present in TDLNs and the ratio of GATA-3/T-bet lymphoid infiltrates in the corresponding tumor samples (Fig. 2A). We then evaluated the clinical relevance of basophil recruitment into TDLNs in 36 patients for which survival data were available. Patient characteristics grouped by the median value of the percentage of basophils in TDLNs are reported in Table 1. Median, 1-year, and 2-year DFS was 16.7 months, 67% and 44% for patients with a percentage of basophils inferior to the median value (n = 18), and 10.6 months, 33% and 22% for patients with a percentage of basophils superior to the median value (n = 18; P = 0.033; Fig. 2B). Multivariate analysis stratifying for tumor stage, grading, size, site, surgical resection margins, and treatment confirmed that the percentage of basophils in TDLNs was independently predictive of DFS (P = 0.02, HR = 11.07, range 1.38–88.60) and OS (P = 0.04, HR = 8.51, range 1.04–69.33; Supplementary Table S1).

Collectively, the presence of basophils in TDLNs correlates with predominant Th2 inflammation in PDAC and disease recurrence, and the percentage of basophils in TDLNs was identified as an independent prognostic factor of the survival of patients after surgery.

Basophil-deficient mice after orthotopic transplantation with tumor cells from KP+C mice did not fully develop the tumor.

To support the role of basophils in PDAC progression, we moved to a mouse model suitable for basophil depletion. We first verified whether the features of Th2 inflammation described for human PDAC were present in a genetically engineered mouse model of spontaneous PDAC. Tumor samples from KP+C mice [carrying the KrasG12D mutation (21) and p53 loss (23)] were analyzed by both in situ RNA hybridization for Gata-3 and T-bet–expressing cells within the stroma and quantitative RT-PCR in total pancreas. In agreement with the human data (9), we found that the number of Gata-3–highly exceeded that of T-bet–expressing lymphoid cells (Fig. 3A). Furthermore, the ratio of Gata-3 and T-bet expression was significantly higher in KP+C mice compared with Kras mice (carrying the KrasG12D mutation only) at 9 weeks of age or WT mice (Fig. 3B), both used as negative controls. Tslp expression was increased in pancreata from KP+C compared with WT and Kras mice, although in the latter case, the increase did not reach statistical significance (Fig. 3C). Basophils, identified by IHC using the murine basophil–specific marker (i.e., MCP-8; ref. 30), were found significantly enriched in tumor-associated lymph nodes from KP+C when compared with peripancreatic lymph nodes from Kras mice (Fig. 3D and E). Together, these results suggest that the KP+C mouse model of spontaneous PDAC recapitulates the features, comprising the recruitment of basophils into tumor-associated lymph nodes, of predominant Th2 responses of the human disease.

We then took advantage of a recently developed mouse model in which basophils are constitutively depleted (i.e., Mcpt8Cre mice; ref. 20). Mcpt8Cre and WT mice were orthotopically transplanted with tumor cells from lines established from KP+C mice and sacrificed after 1 or 8 weeks. At 1 week after implant, hematoyxlin and eosin staining showed tumor development in the area of tumor cell implant in both Mcpt8Cre and WT mice (Fig. 3F), with 100% of tumor take in both mouse strains (Fig. 3J). At 8 weeks after implant, tumor development was observed in WT, but not in Mcpt8Cre mice (Fig. 3G), and, strikingly, tumor was present in 80% for WT animals and in none of basophil-deficient mice (P = 0.0012; Fig. 3I). When we looked for the presence of basophils in peripancreatic lymph nodes, we found basophil recruitment into tumor-associated lymph nodes of tumor-bearing WT (Fig. 3H, top, and I) but as expected, not Mcpt8Cre (Fig. 3H, bottom, and I) mice.

Collectively, similar to human disease basophils are recruited to tumor-associated lymph nodes in mice with spontaneous development of PDAC, and basophil depletion highly impacts on full tumor development.

T cell–derived IL3 in TDLNs induces basophil activation

Basophils readily generate/release IL4 in response to various stimuli, including IL3 both used as priming cytokine and, although at lower levels, alone (31, 32). In addition, it has been recently reported that TSLP-activated DCs prime naïve CD4+ T cells to produce IL3 in early stages of Th2 differentiation (33). We found a significantly increased IL3 mRNA expression in TDLNs compared with non-TDLNs (Fig. 4A) and identified T cells purified from TDLNs as a source of IL3 in 10 of 11 samples analyzed (Fig. 4B). Interestingly, the levels of IL3 significantly correlated with those of IL4 in corresponding TDLNs (Fig. 4C). Next, we performed in vitro experiments to model a possible mechanism of basophil activation in vivo. We purified myeloid DCs from the blood of HDs, as described in ref. 9 and conditioned them with TSLP or the supernatant of CAFs either untreated or treated with TNPx and IL1 (i.e., cytokines that drive TSLP secretion by CAFs). DCs untreated or treated with lipopolysaccharide were used as controls. Naïve T cells were then activated in vitro, with DCs conditioned with the different stimuli. In agreement with ref. 33, we confirmed IL3 secretion by T cells activated with TSLP-conditioned DCs and, importantly, we found that CAF-conditioned DCs induced IL3 secretion by T cells, as early as after 4 days of culture (i.e., time point at which T cells do not secrete IL4, not shown), that was significantly inhibited by an anti-TSLP antibody (Fig. 4D). Then, we used the supernatant of IL3-producing T cells to activate basophils isolated from the blood of HDs. Basophil
activation was determined by flow cytometry as CD203c upregulation, as reported in refs. 26, 27. Both recombinant IL3 (Fig. 4E) and T cell–derived IL3 (Fig. 4F and G) caused CD203c upregulation (even when IL3 was present in the supernatant at a very low concentration) that was inhibited by anti-IL3 antibody. Finally, the level of basophil activation induced in vitro by T cell–derived IL3 was confirmed ex vivo by CD203c upregulation within the basophil-enriched fraction (c-kit+/CD123+) purified from TDLNs (Fig. 4H).

Together, although we cannot directly correlate CD203c upregulation with IL4 secretion, these data strongly suggest that basophils are activated in vivo by IL3, which is induced within TDLNs in T cells by CAF-derived TSLP-conditioned DCs.

Monocytes in TDLNs recruit basophils through CCL7/MCP3

CCL7/MCP3 is a basophil chemok attractant (34), which was recently identified as the DC-derived chemokine mediating recruitment of IL4+ basophils to the lymph node in a model of Th2 response (35). We found that CCL7/MCP3 expression was significantly increased in TDLNs compared with non-TDLNs (Fig. 5A). To identify the cellular source of CCL7/MCP3 in TDLNs, we treated in vitro myeloid DCs isolated from peripheral blood of HDs with CAF supernatants, but we did not observe CCL7/MCP3 induction and/or secretion (data not shown).

However, CCL7/MCP3 was strongly induced and secreted by similarly treated CD14+ monocytes (Fig. 5B and C). In addition, monocytes treated with CAF supernatants also expressed thymus and activation-regulated chemokine (CCL17/TARC; Fig. 5B), suggesting differentiation towards an alternative activated M2 phenotype (36). Importantly, CCL7/MCP3 and CCL17/TARC coexpressing monocytes (CD14+CD11c+) could be purified ex vivo from TDLNs (Fig. 5D). In agreement with the identification of CCL7/MCP3–expressing monocytes in TDLNs, we found by IHC that basophils localized in close contact with CD11c+ and CD14+ cells (Fig. 5E) immediately after their extravasation (Fig. 5E, top left) and within the medulla (Fig. 5E, top right) in the proximity of sinusoids (Fig. 5E, bottom). Finally, we performed in vitro migration experiments of basophils in the presence of the supernatant of CAF-conditioned monocytes. As shown in Fig. 5F, we observed basophils migration in the presence of the supernatant of activated, but not resting, CAFs, and basophil migration was inhibited by anti-CCL7/MCP3. As the inhibition was partial, we investigated the expression in monocytes treated with CAF supernatants of other known basophil chemotactants (37). We found that CCL24/ eotaxin-2, but not CCL26/ eotaxin-3 and CCL11/ eotaxin-1, was significantly induced (Supplementary Fig. S1A). In agreement, CCL24/ eotaxin-2 expression was also significantly increased in TDLNs compared with non-TDLNs (Supplementary Fig. S1B) and expressed in CD14+CD11c+ cells isolated from TDLNs (Supplementary Fig. S1C). However, in basophil migration experiments, we did not observe inhibition with anti-CCL24 antibody (Supplementary Fig. S1D).

Collectively, these data support a role of monocytes conditioned by activated tumor stroma for basophil recruitment into TDLNs that partially depends on the secretion of CCL7/MCP3.

Discussion

In this study, we report a relevant role for basophils in PDAC progression. Indeed, the percentage of basophils present in TDLNs was identified as an independent prognostic factor of survival after surgery in PDAC patients, and basophil-deficient mice were protected from full tumor development.

To our knowledge, this is the first demonstration of a role for basophils as accessory cells, exerting clinically relevant tumor-promoting functions within TDLNs. The proposed model of how basophils are recruited and activated in TDLNs in the context of PDAC and how they cooperate with TSLP-conditioned DCs for development of Th2 responses is depicted in Fig. 6. As previously reported (9), DCs are endowed with Th2-polarizing capabilities in the tumor microenvironment by CAF-derived TSLP (Fig. 6A), and TSLP-conditioned DCs migrate to TDLNs where they prime T cells for early IL-3 secretion (Fig. 6B). Monocytes, which are driven to differentiate towards a M2 type by the supernatant of activated CAFs (Fig. 6C), release the basophil chemotactant CCL7/MCP3 (Fig. 6D) and basophils recruited into TDLNs are activated by T cell–derived IL3 and express the IL4 necessary for Th2 stabilization (Fig. 6E).
Basophils represent less than 1% of peripheral blood leukocytes and share several characteristics with tissue-resident mast cells (17, 18). In recent years and because of development of tools for their functional analysis and of genetically engineered mice deficient only in basophils, a nonredundant role for basophils in regulation of acquired immunity, protective immunity to helmiths, allergy, and autoimmunity has been established (17, 18, 38).

We believe that a nonredundant role for basophils also applies to PDAC. Mast cells have been extensively studied in PDAC, where the degree of their infiltration was identified as a predictive marker of patient survival (39–42) and correlated with lymph node metastases, tumor grade, microvessel density, and lymphatic and microvascular invasion (39, 41, 42); although their relevance in pancreatic tumorigenesis in animal models yielded somehow conflicting results, depending on the model used (40, 43). Basophils in our study were the cells mainly responsible for the presence of IL4 in TDLNs of patients, where their percentage significantly correlated with IL4 expression and the ratio of Th2/Th1 lymphoid cells in the corresponding tumor. The experiments performed in basophil-deficient mice indicated that basophils are apparently dispensable for tumor take. Indeed, we did not observe differences in tumor development between basophil-deficient and WT mice at 1 week after PDAC cell transplant. Notably, basophils were absolutely required for long-term tumor establishment; indeed, at 8 weeks after PDAC cell transplant, the tumor developed in 80% of WT mice, while, strikingly, we did not observe tumor in any of the basophil-deficient mice. Whether basophil-derived IL4 is required for full tumor development and progression or whether basophil-deficient mice rejected the tumor needs to be established in future studies. Together, data from the reported literature (5, 7) and this study point to distinct roles for mast cells and basophils in PDAC, where mast cells within the tumor favor angiogenesis, tumor growth, and invasion, whereas basophils within TDLNs favor tumor progression, as it is possible that once the mechanisms of basophil recruitment and activation have been established, basophil-derived IL4 and IL3 contribute to reinforce both basophil activation through an autocrine loop and the M2 polarization in TDLNs.

In summary, the data reported here add further complexity to the cross-talk within the tumor microenvironment that leads to predominant Th2 inflammation in PDAC by the identification of new cellular (i.e., the basophils) and molecular (i.e., IL4, IL3, and CCL7/MCP3) components present in TDLNs, possibly amenable in the future to therapeutic targeting.

Disclosure of Potential Conflicts of Interest
M. Reni has received speakers bureau honoraria from Celgene and is a consultant/advisory board member for Baxalta, Boehringer, Celgene, Clovis, Genentech, Lilly, and Merck-Serono. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: L. De Monte, M.P. Protti
Development of methodology: L. De Monte, E. Brunetto, H. Recalde, H. Algul, C. Doglioni
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. De Monte, S. Woermann, S. Heltai, M. Reni, A. Mondino, M. Falcioni, F. Alleotti, G. Balzano, H. Algul, C. Doglioni
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. De Monte, S. Woermann, M. Reni, A.M. Paganoni, C. Doglioni, M.P. Protti
Writing, review, and/or revision of the manuscript: L. De Monte, S. Woermann, C. Doglioni, M.P. Protti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Woermann, S. Heltai, G. Magliacane, F. Alleotti, M.P. Protti
Study supervision: L. De Monte, M.P. Protti

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References

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Lucia De Monte, Sonja Wörmann, Emanuela Brunetto, et al.

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