Basophil recruitment into tumor draining lymph nodes correlates with Th2 inflammation and reduced survival in pancreatic cancer patients

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ABSTRACT

In pancreatic ductal adenocarcinomas (PDAC), lymphoid infiltrates comprised mainly of T helper 2 (Th2) cells predict a poor survival outcome in patients. IL-4 signaling has been suggested to stabilize the Th2 phenotype in this setting, but the cellular source of IL-4 in PDAC is unclear. Here we show that basophils expressing IL-4 are enriched in tumor-draining lymph nodes (TDLNs) 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. 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 IL-3. 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 illuminating a key element of the immune milieu of pancreatic cancer.

INTRODUCTION

PDAC is a very aggressive disease with dismal prognosis (1). The crosstalk within the tumor microenvironment among different cellular components has 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 patients' survival after surgery (9). We identified the thymic stromal lymphopoietin (TSLP), which is produced by activated cancer associated fibroblasts (CAFs), as a key cytokine for conditioning myeloid dendritic cells (DCs) with Th2 polarizing capability that reside within the tumor stroma and in TDLNs (9, 10).

How are Th2 immune responses 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 IL-4 (12), the IL-4/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 IL-4 had prompted the search for identifying accessory cells that provide in vivo the early innate source of IL-4.

Proposed sources of IL-4 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 IL-4 after their transient recruitment into draining LNs 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 IL-4 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.

MATERIALS AND METHODS

Patients' 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 LN specimens were collected at surgery. TDLNs were collected from fresh surgical specimens and non-TDLNs from the distal mesentery. 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 (IPMN) (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) had 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 LNs

CAFs obtained by outgrowth from tumor specimens were characterized and activated for TSLP secretion as described in (9). Samples of TDLN and non-TDLNs were either immediately frozen in RNA-later (Ambion) for RNA extraction or freshly processed to obtain single cell suspension for 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 from non-T cells by negative selection using PanT cell isolation kit (Miltenyi Biotec) while

CD14 microbeads (Miltenyi Biotec) were used to positively select monocytes/macrophages.

Mice and mice-derived tumor cell lines

Mcpt8Cre mice (20) were kindly provided by Prof. David Voehringer (University of Erlangen). LSL-Kras^{G12D} knockin (21), Ptf1a-cre^{ex1} (22) and p53$^{F/F}$ strains (23) were interbred to obtain compound mutant LSL-Kras^{G12D}; p53$^{F/F}$; Ptf1a-cre^{ex1} termed *KP$^{\Delta}$C* mice. LSL-Kras^{G12D} knockin and Ptf1a-cre^{ex1} were interbred to obtain compound mutant LSL-Kras^{G12D}; Ptf1a-cre^{ex1} 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 WT. All procedures were conform to the regulatory standards and approved by the Regierung von Oberbayern (Munich, Germany). Primary tumor cell lines were established from *KP$^{\Delta}$C* pancreatic tumors and cultured until passage 6-8 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 RNAeasy mini kit (Quiagen), according to the manufacturers' instructions. 50-100 ng cDNA/sample obtained with High-Capacity cDNA reverse transcription kit (Applied Biosystems) were used for qRT-PCR. Murine cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). Taqman primers specific for human IL-4, IL-3, 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 previously described (9, 22). Data were

normalized to HGPRT or β-actin levels for human- and to endogenous cyclophillin for mice mRNA, respectively. Fold induction was calculated by the $2^{-\Delta\Delta Ct}$ method.

In situ hybridization

The labeling of complementary RNA strands to localize specific IL-4 expression in LNs from PDAC patients and T-bet and GATA-3 RNA sequences in pancreatic tumors from *KP$^{\Delta}$C* mice was performed applying the RNAscope 2.0 FFPE Assay (Advanced Cell Diagnostics), according to the manufacturers protocol. Microscopic observation was performed using Nikon Eclipse 80i and images digitalized using Aperio ScanScope (Nikon Instruments SpA).

Orthotopic Transplantation Model

$1x10^6$ cells from primary tumor cell lines established from *KP$^{\Delta}$C* mice were orthotopically transplanted into 8-week-old *Mcpt8Cre* and WT mice. Mice were sacrificed at the indicated time points.

Immunohistochemistry

Immunohistochemistry was performed on tissue sections from tumor and LN specimens as detailed in (24). The following antibodies (Abs) were used: anti-pro-MBP1 (Biolegend, Clone J175-7D4), anti-T-bet (Santa Cruz Biotechnology, Inc.), anti-GATA-3 (R&D), anti-CD11c (Novocastra, NCL-CD11c-536) and anti-CD14 (Novocastra). Quantification of GATA-3^+ and T-bet^+ lymphoid cells was performed as described in (9). For pro-MBP1, up to 4 representative areas per sample within the LN medulla were selected; pro-$MBP1^+$ cells were quantified with the Aperio ImageScope Nuclear Algorithm and the average % of the pro-MPB1 positive cells of

the analyzed regions was reported. Sections used for IL-4 in situ hybridization were stained with anti-pro-MBP1 Ab 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 LNs were stained with mMCP-8 (TUG8 clone) rat anti-mouse Ab (Biolegend) utilizing an anti-rat DAB polymer detection system (Rat on Mouse polymer Biocare). 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 (HDs). Basophil enrichment was performed as described in (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), CD203c-APC (Miltenyi Biotec), and FcεRI-APC, c-kit-PE or c-kit-BV421 (BD) specific Abs. Naïve $CD4^+$ T cells were stained with CD4 and CD45RA specific Abs (BD). CD3-APC, CD4-PE, CD8-FITC Abs (BD) were used to quantify T cells recovered from the LNs. Sample acquisition and analysis were performed with FACSCanto instrument and FlowJo software (LLC), respectively.

Myeloid DC isolation and activation

97-99% pure myeloid DCs were obtained as described in (9) and cultured for 24 hours at $1x10^6$/ml in 96-well plates in IMDM 2% FBS ± the following stimuli: 25 ng/ml TSLP (R&D), 1 mg/ml *E.Coli* LPS (Sigma) and supernatants of untreated or TNF-α+IL1-β-treated CAFs. In inhibition experiments 2μg/ml anti-TSLP or isotype control Abs (R&D) were added.

DC:T cell co-culture

≥95% pure naïve $CD4^+$ T cells were obtained with Naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec). Activated DCs were added to naïve $CD4^+$ T cells at a 1:5 ratio in 96-well plates. After 2, 3, 4, 5 days the cells were collected, washed and re-stimulated for 24 hours with anti-human CD3/CD28 Abs-coupled microbeads (Miltenyi Biotec). IL-3 release in the supernatant was measured by ELISA (R&D). Day 4 was identified as the optimal condition and used for the basophil activation experiments.

T-cell derived IL-3-dependent basophil activation

$1x10^6$/ml/well basophils were plated in 96-well plates and cultured for 16 hours in IMDM 10% FBS ± the following stimuli: 5 ng/ml rIL-3 (R&D) and supernatants from T cells co-cultured with DCs (containing 60-200 pg/ml IL-3). IL-3 neutralizing or isotype control Abs (R&D) were added at 1 μg/ml. Basophil activation was measured by FACS analysis by quantitating CD203c expression, as reported in (26, 27).

Basophil migration assay

$1x10^6$/ml $CD14^+$ monocytes were plated in 24-well plates in IMDM 5% FBS ± untreated or activated CAF supernatants. After 4 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) and stored at -20°C for further use. Basophil migration was performed as described in (28) with minor modifications. Briefly, $2x10^4$ basophils were added to the upper chamber and monocyte supernatants placed in the lower chamber. Anti-CCL7/MCP3 neutralizing or isotype control Abs (R&D) 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 Abs (Miltenyi Biotec). Each sample was re-suspended 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 Ab (R&D).

Statistical analysis

To test the stochastic order between non normal 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's *t* test for normal data were applied. Correlation between variables was determined by means of nonparametric two-tailed Spearman *r* test. Pearson's chi-squared 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 and overall survival. 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).

RESULTS

IL-4 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 towards Th2 (9). However, as IL-4 exerts a key role to stabilize the Th2 phenotype (11, 13), we looked for its expression in PDAC surgical samples and TDLNs. While IL-4 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 IL-4 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 IL-4 secretion (17, 18). To assess their presence in TDLNs we used flow cytometry analysis (Fig. 1B) and immunohistochemistry (Fig. 1C). By both techniques we were able to identify basophils, which were located in the proximity of the sinusoids within the medulla (Fig. 1C) and whose percentages were significantly increased in TDLNs compared with non-TDLNs (Fig. 1D). Interestingly, the percentages of basophils in TDLNs from PDAC patients were significantly higher than those found in TDLNs from patients bearing pancreatic benign neoplasms (Fig. 1E). To verify whether basophils were responsible for the presence of IL-4, we purified the basophil positive and negative fractions from TDLNs. We found significant higher IL-4 expression in the basophil-enriched compared with the basophil-negative fraction (Fig. 1F). We then performed in situ hybridization for IL-4 mRNA expression followed by basophils specific staining (i.e., pro-MBP1) (29) by immunohistochemistry. In agreement with IL-4 expression in the basophil-enriched fraction, we confirmed that

the majority of IL-4 expressing cells in TDLNs were indeed basophils (Fig. 1G) and that their percentage significantly correlated with IL-4 expression in corresponding TDLNs (Fig. 1H).

Collectively, although we cannot exclude that other cells expressing low levels of IL-4 are also present, these data indicate that the main cellular sources of IL-4, which is necessary to stabilize the Th2 phenotype in PDAC, are basophils recruited into TDLNs.

The percentage of basophils in TDLNs significantly correlates with predominant Th2 inflammation and predicts survival after surgery in PDAC patients

To determine the possible correlation between predominant Th2 cell infiltrates 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 analyzed in (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 % 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. Patients' characteristics grouped by the median value of the % of basophils in TDLNs are reported in Table 1. Median, 1-year and 2-year disease free survival was 16.7 months, 67% and 44% for patients with a % of basophils inferior to the median value (n=18) and 10.6 months, 33% and 22% for patients with a % 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 % of basophils in

TDLNs was independently predictive of disease free survival (p=0.02, HR=11.07, range 1.38-88.60) and overall survival (p=0.04, HR=8.51, 1.04-69.33)(Supplementary Table 1).

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 patients' survival after surgery.

Basophil-deficient mice after orthotopic transplantation with tumor cells from *KP$^{\Delta}$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$^{\Delta}$C* mice (carrying the *Kras*^{G12D} 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 those of T-bet- expressing lymphoid cells (Fig. 3A). Furthermore, the ratio of GATA-3 and T-bet expression was significantly higher in *KP$^{\Delta}$C* mice compared with *Kras* mice (carrying the *Kras*^{G12D} 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$^{\Delta}$C* compared with WT and *Kras* mice, although in the latter case the increase did not reach statistical significance (Fig. 3C). Basophils, identified by immunohistochemistry using the murine basophils specific marker (i.e., MCP-8) (30), were found significantly

enriched in tumor-associated LNs from *KP$^{\Delta}$C* when compared with peripancreatic LNs from *Kras* mice (Fig. 3D-E). Together, these results suggest that the *KP$^{\Delta}$C* mouse model of spontaneous PDAC recapitulates the features, comprising the recruitment of basophils into tumor-associated LNs, 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)(20). *Mcpt8Cre* and WT mice were orthotopically transplanted with tumor cells from lines established from *KP$^{\Delta}$C* mice and sacrificed after 1 or 8 weeks. At 1 week after implant hematoxylin and eosin (H&E) 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. 3J). When we looked for the presence of basophils in peripancreatic LNs we found basophil recruitment into tumor-associated LNs of tumor bearing WT (Fig. 3H, upper panel and Fig. 3I) but, as expected, not *Mcpt8Cre* (Fig. 3H, lower panel and Fig. 3I) mice.

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

T-cell-derived IL-3 in TDLNs induces basophil activation

Basophils readily generate/release IL-4 in response to various stimuli, including IL-3 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 IL-3 in early stages of Th2 differentiation (33). We found a significantly increased IL-3 mRNA expression in TDLNs compared with non-TDLNs (Fig. 4A) and identified T cells purified from TDLNs as a source of IL-3 in 10 out of 11 samples analyzed (Fig. 4B). Interestingly, the levels of IL-3 significantly correlated with those of IL-4 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 (9) and conditioned them with TSLP or the supernatant of CAFs either untreated or treated with TNF-α and IL-1 (i.e., cytokines that drive TSLP secretion by CAFs). DCs untreated or treated with LPS were used as controls. Naïve T cells were then activated in vitro with DCs conditioned with the different stimuli. In agreement with (33), we confirmed IL-3 secretion by T cells activated with TSLP-conditioned DCs and, importantly, we found that CAF-conditioned DCs induced IL-3 secretion by T cells, as early as after 4 d of culture (i.e., time point at which T cells do not secrete IL-4, not shown), that was significantly inhibited by an anti-TSLP Ab (Fig. 4D). Then, we used the supernatant of IL-3-producing T cells to activate basophils isolated from the blood of HDs. Basophil activation was determined by flow cytometry as CD203c up-regulation, as reported in (26, 27). Both recombinant IL-3 (Fig. 4E) and T cell-derived IL-3 (Fig. 4F-G) caused CD203c up-regulation (even when IL-3 was present in the supernatant at very low concentration) that was inhibited by anti-IL-3 Ab. Finally, the level of basophil activation induced in vitro by T-cell derived IL-3 was confirmed ex-vivo by CD203c up-regulation within the basophil-enriched fraction (c-kit$^-$CD123$^+$) purified from TDLNs (Fig. 4H).

Together, although we can not directly correlate CD203c upregulation with IL-4 secretion, these data strongly suggest that basophil are activated in vivo by IL-3, 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 chemoattractant (34), which was recently identified as the DC-derived chemokine mediating recruitment of IL-4$^+$ basophils to the LN 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 CD14$^+$ monocytes similarly treated (Fig. 5B-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 co-expressing 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 immunohistochemistry that basophils localized in close contact with CD11c$^+$ and CD14$^+$ cells (Fig. 5E), immediately after their extravasation (Fig. 5E, upper left) and within the medulla (Fig. 5E, upper right) in the proximity of sinusoids (Fig. 5E, lower panels). Lastly, 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 inhibition was partial, we investigated the expression in monocytes treated with CAF supernatants of other known basophil chemoattractants (37). We found that CCL24/eotaxin-2 but not CCL26/eotaxin-3 and CCL11/eaotaxin-1 was significantly induced (Supplementary Fig 1A). In agreement, CCL24/eotaxin-2 expression was also significantly increased in TDLNs compared with non-TDLNs (Supplementary Fig. 1B) and expressed in $CD14^{+}CD11c^{+}$ cells isolated from TDLNs (Supplementary Fig. 1C). However, in basophil migration experiments we did not observed inhibition with anti-CCL24 Ab (Supplementary Fig. 1D).

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 (A), TSLP-conditioned DCs migrate to TDLNs were they prime T cells for early IL-3 secretion (B), monocytes, which are driven to differentiate towards a M2-type by the supernatant of activated CAFs (C), release the basophil chemoattractant CCL7/MCP3 (D) and basophils recruited into TDLNs are activated by T-cell-derived IL-3 and express the IL-4 necessary for Th2 stabilization (E).

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 non-redundant role for basophils in regulation of acquired immunity, protective immunity to helminths, allergy and autoimmunity has been established (17, 18, 38).

We believe that a non-redundant 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 patients' survival (39-42) and correlated with LN

metastases, tumor grade, microvessel density and lymphatic and microvascular invasion (39, 41, 42); while 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 IL-4 in patients' TDLNs where their percentage significantly correlated with IL-4 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 basophils derived IL-4 is required for full tumor development and progression or whether basophil-deficient mice rejected the tumor need to be established in future studies. Together, data from the reported literature (5, 7) and the present study point to distinct roles for mast cells and basophils in PDAC where mast cells within the tumor favor angiogenesis, tumor growth and invasion while basophils within TDLNs favor tumor progression possibly through the establishment of predominant Th2 inflammation.

Heterogeneity within the murine basophil cell lineage has been recently proposed (44). Although it has been clearly demonstrated that IL-3 is a key regulator of basophil development in the context of some stimuli (44), TSLP was shown to regulate basophil development and to induce peripheral basophilia in both IL-3-IL-3R-sufficient and -deficient environments (45), suggesting a non-redundant role for TSLP in basophils development and activation. In addition, genome-wide

transcriptional profiling and functional analyses identified heterogeneity between IL-3-elicited versus TSLP-elicited basophils (45). We believe that, in our system, IL-3 and TSLP exert complementary functions in basophil activation (Fig. 6): indeed, ex-vivo and in vitro experiments strongly point to a direct role for T cell-derived IL-3 while CAF-derived TSLP would exert an indirect role through DC conditioning. Whereas a direct role for TSLP in eliciting basophil functions seems to be excluded because, at difference with what has been reported in (45), we did not detect systemic basophilia in PDAC patients (unpublished observations) and basophil activation by CAF-derived TSLP (unpublished observations).

Another interesting finding of our study is the presence within TDLNs of bona fide M2-type cells (i.e., CCL17/TARC expressing monocytes) responsible for the release of CCL7/MCP3. Interestingly, monocyte differentiation towards M2 was induced in vitro by CAF supernatants and we envisage that in vivo CAF derived factor(s) could reach the TDLNs through the conduit system from the afferent lymphatic vessels (46). Preliminary data do not support a role for TSLP in driving CCL7/MCP3 secretion by M2 monocytes, thus future investigation would possibly identify the CAFs-derived factor(s) implicated. In addition, it has been shown that IL-3 synergizes with basophil-derived IL-4 and IL-13 to promote M2 differentiation (47) and that activated basophils can secrete IL-3 (48). It is possible that once the mechanisms of basophil recruitment and activation have been established, basophil-derived IL-4 and IL-3 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., IL-4, IL-3

and CCL7/MCP3) components present in TDLNs possibly amenable in the future to therapeutic targeting.

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

Variable	% pro-MBP1 < 0.44 (n=18)	% pro-MBP1> 0.44 (n=18)
Gender: female	11 (61%)	10 (56%)
Median age	67	62,5
Tumor site: head	14 (78%)	18 (100%)
Tumor size > 3 cm	3 (17%)	5 (28%)
Stage IIB/III	16 (89%)	13 (72%)
Grade 3	6 (34%)	5 (28%)
Karnofsky >80	12 (67%)	12 (67%)
Surgical margin R1	5 (28%)	10 (56%)
Treatment: PEFG[a]	8 (45%)	11 (61%)

[a]Patients who had R0 or R1 resection of a stage IB-III PDAC, aged 18-75 years and Karnofsky Performance Status >60, were eligible for adjuvant therapy. The patients were required to have post-operative 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 (49) and 19 patients received the PEFG regimen consisting of cisplatin, epirubicin, gemcitabine and 5-fluorouracil (50). In both cases, chemotherapy was administered for 3 months followed by chemo-radiation (50).

FIGURE LEGENDS

Figure 1. IL-4 secreting basophils are significantly increased in TDLNs compared with non-TDLNs of PDAC patients. **A,** IL-4 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 ($CD123^{+}FcεRI^{+}c\text{-}kit^{-}$) in TDLNs and non-TDLNs of patient #54. **C,** representative (n=18) immunohistochemical analysis for basophil (pro-MBP1) in TDLNs and non-TDLNs of patient #3. Arrows indicate pro-MBP1 positive cells. **D,** quantification of the % of basophils in the 18 paired samples tested. Significance was determined using one-tailed Wilcoxon matched-pairs signed rank test (p=0.0009). **E**, quantification of the % 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 was p=0.002, p=0.007 and p=0.005, respectively. **F,** IL-4 mRNA expression in basophil-enriched and non-enriched cell populations (n=5). Significance was determined using one-tailed Wilcoxon matched-pairs signed rank test (p=0.0313). **G,** representative (n=4) in situ hybridization for IL-4 mRNA in TDLNs followed by immunohistochemical analysis for pro-MBP1 of patient #6. Red= pro-MBP1, brown= IL-4. Arrows indicate double positive cells. **H,** scatter plot of the % of basophils and IL-4 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).

Figure 2. The percentage of basophils in TDLNs correlates with the ratio of $GATA\text{-}3^{+}/T\text{-}bet^{+}$ lymphoid infiltrate in the tumor and predicts disease free survival after

surgery in stage IB-III PDAC patients. **A,** scatter plot of the % of basophils in TDLNs and the ratio of GATA-3^{+}/T-bet^{+} lymphoid cells in the corresponding tumors (n=47). Significance was determined using nonparametric two-tailed Spearman *r* test (r=0.3046). **B,** Kaplan-Meier curve for disease-free survival by median % of basophils. Significance was evaluated by the Log-rank (Mantel-Cox) test. Survival significantly decreased as a function of a % of basophils in the TDLNs superior to the median value.

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 *KP$^{\Delta}$C* mice (n=2). **B-C**, ratio of the GATA-3/T-bet (**B**) and TSLP (**C**) mRNA expression in pancreata from *KP$^{\Delta}$C*, *Kras* at 9 weeks of age and WT mice. Significance was determined by two-tailed Mann Whitney test. GATA-3/T-bet: *KP$^{\Delta}$C vs Kras* (p=0.017) and *KP$^{\Delta}$C vs* WT (p=0.035). TSLP: *KP$^{\Delta}$C vs* WT (p=0.021), *Kras vs* WT (p=0.032). **D,** representative staining for MCP-8 (murine basophil marker) in tumor-associated LNs in tumor bearing *KP$^{\Delta}$C* mice (n=8) and peripancreatic LNs in Kras mice at 9 weeks of age (n=4). **E**, quantification of the % of basophils from (D). Significance was determined by one-tailed Mann Whitney test (p=0.004). **F,** representative H&E staining in WT (n=5) and *Mcpt8Cre* (n=4) mice at 1 week after orthotopic injection of tumor cells from *KP$^{\Delta}$C* mice. **G,** representative H&E staining in WT (n=4) and *Mcpt8Cre* (n=4) mice at 8 week after orthotopic injection of tumor cells from *KP$^{\Delta}$C* mice. **H,** MCP-8 staining for basophils detection in tumor-associated LNs in WT mice and in peripancreatic LNs in *Mcpt8Cre* mice. **I**, quantification of the % of basophils from (H). Significance was determined by one-tailed Mann Whitney test (p=0.009). **J,** % 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's chi-squared test (p=0.0012).

Figure 4. T cell-derived IL-3 induces basophil activation. **A,** IL-3 mRNA expression in paired TDLNs and non-TDLNs (n=13). Significance was determined using one-tailed Wilcoxon matched-pairs signed rank test (p=0.0003). **B,** IL-3 mRNA expression in T cells purified from TDLNs of the indicated PDAC patients. **C,** scatter plot of IL-3 and IL-4 mRNA expression in TDLNs (n=9). Significance of the correlation was determined using nonparametric two-tailed Spearman r test (r=0.8167). **D,** in vitro IL-3 released by T cells after 4 days activation with myeloid DCs treated as indicated. Where reported anti-TSLP or isotype control Abs were added in the culture. Significance was determined by unpaired, one-tailed Student's *t* test and indicated as: *p<0,05 and **0,001<p<0,01. Representative of 4 experiments performed with 3 different CAFs. (**E-F-G**) Flow cytometry analysis of basophil activation (i.e., CD203c up-regulation) after treatment with ionomycin, as maximal stimulation, recombinant IL-3 (5 ng/ml), used as positive control (**E)**, or two IL-3-containing supernatants of T cells activated with CAF-conditioned DCs (**F-G),** IL-3 quantification in T cell supernatants (**F-G, left panels**). Basophils' activation was inhibited in the presence of anti-IL-3 but not isotype control Abs (**F-G, right panels**). **H,** representative (n=3) ex-vivo up-regulation of CD203c in the basophil-enriched fraction (c-kit$^-$CD123$^+$) from TDLNs of patient #54.

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 LPS were used as controls. **C,** CCL7/MCP3 secretion from blood derived $CD14^+$ monocytes stimulated in vitro for 24 hours ± 1 mg/ml LPS or activated-CAF supernatants. Significance was determined by unpaired, one-tailed Student's *t* test and indicated as: ***0,001<p<0,01. Representative of 2 experiments performed with 2 different CAFs. **D,** CCL7/MCP3 and CCL17/TARC mRNA expression in $CD14^+CD11c^+$ monocytes purified from TDLNs of the indicated PDAC patients. **E,** immunohistochemistry for pro-MBP1 and CD11c (upper panel) and CD14 (lower panel). **Upper panels**: pro-MBP1 (brown) and CD11c (red). **Lower panel**: pro-MBP1 (red) and CD14 (brown). Arrows indicate basophils ($pro\text{-}MBP1^+$) in close proximity with $CD11c^+$ and $CD14^+$ cells. **F,** migration experiments. Basophils were seeded in the upper chamber and the supernatant of $CD14^+$ monocytes, conditioned as indicated (upper panel), in the lower chamber. The numbers of migrated basophils in the absence or in the presence of the anti-CCL7 or an isotype control Ab are reported. Representative of 4 experiments performed with 4 different monocytes/basophils preparations and 4 different CAFs.

Figure 6. Proposed model of cellular and molecular cross-talk leading to basophil recruitment and activation within TDLNs in PDAC. **A,** DCs are activated in the tumor stroma by CAF-derived TSLP. **B,** TSLP-conditioned DCs migrate to TDLNs where they activate T cells to secrete IL-3. **C,** resident monocytes are differentiated towards M2 (i.e., expressing CCL17/TARC) by CAF-derived factor(s) and **D,** secrete CCL7/MCP3 that in turn recruits basophils to TDLNs. **E,** recruited basophils are

activated by T cell derived-IL-3 to release IL-4 that in turn induces STAT6 dependent GATA-3 expression in Th2 cells.

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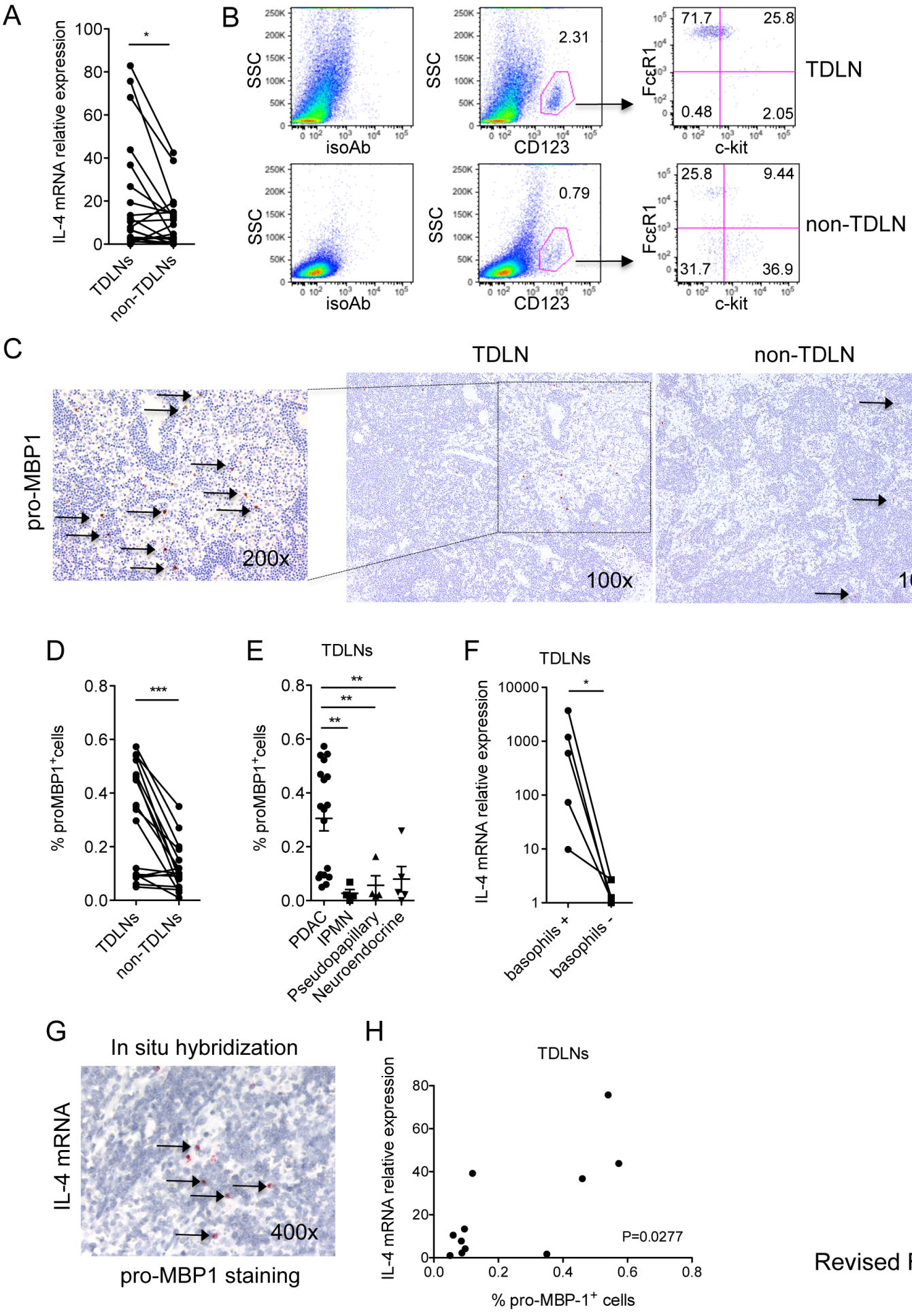

Revised Figure 1

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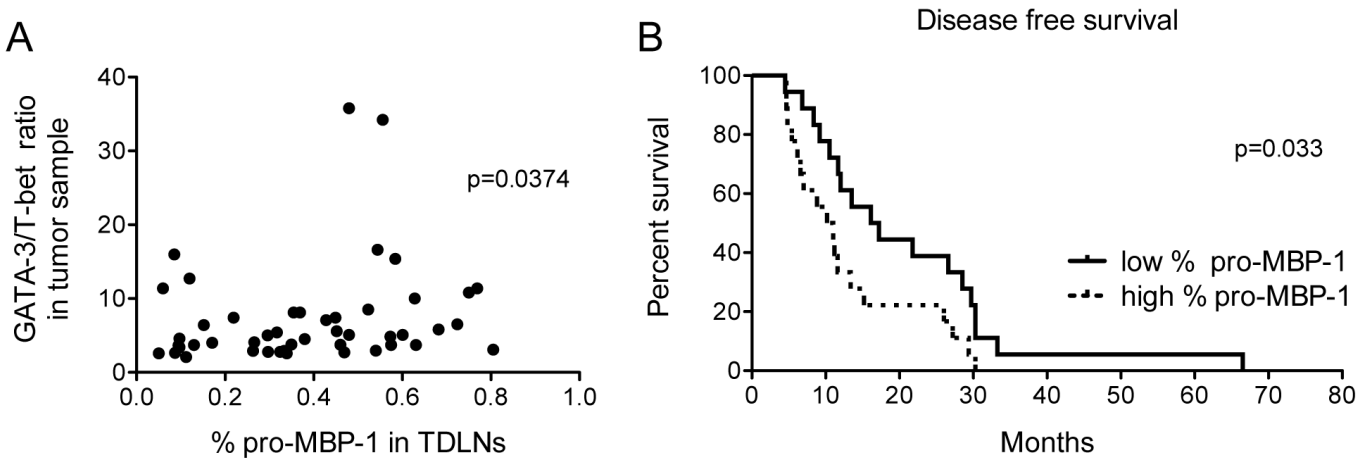

Revised Figure 2

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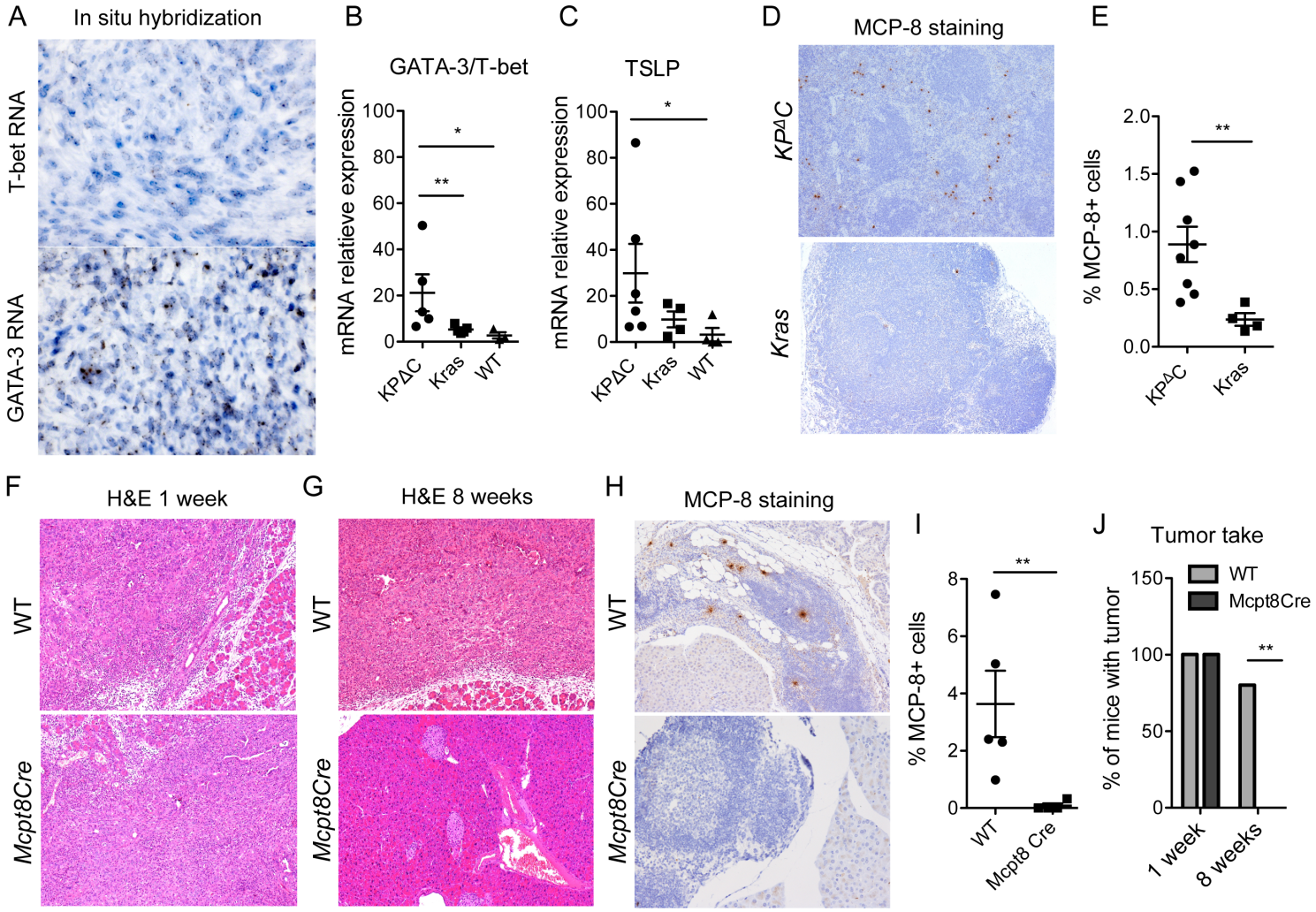

Revised Figure 3

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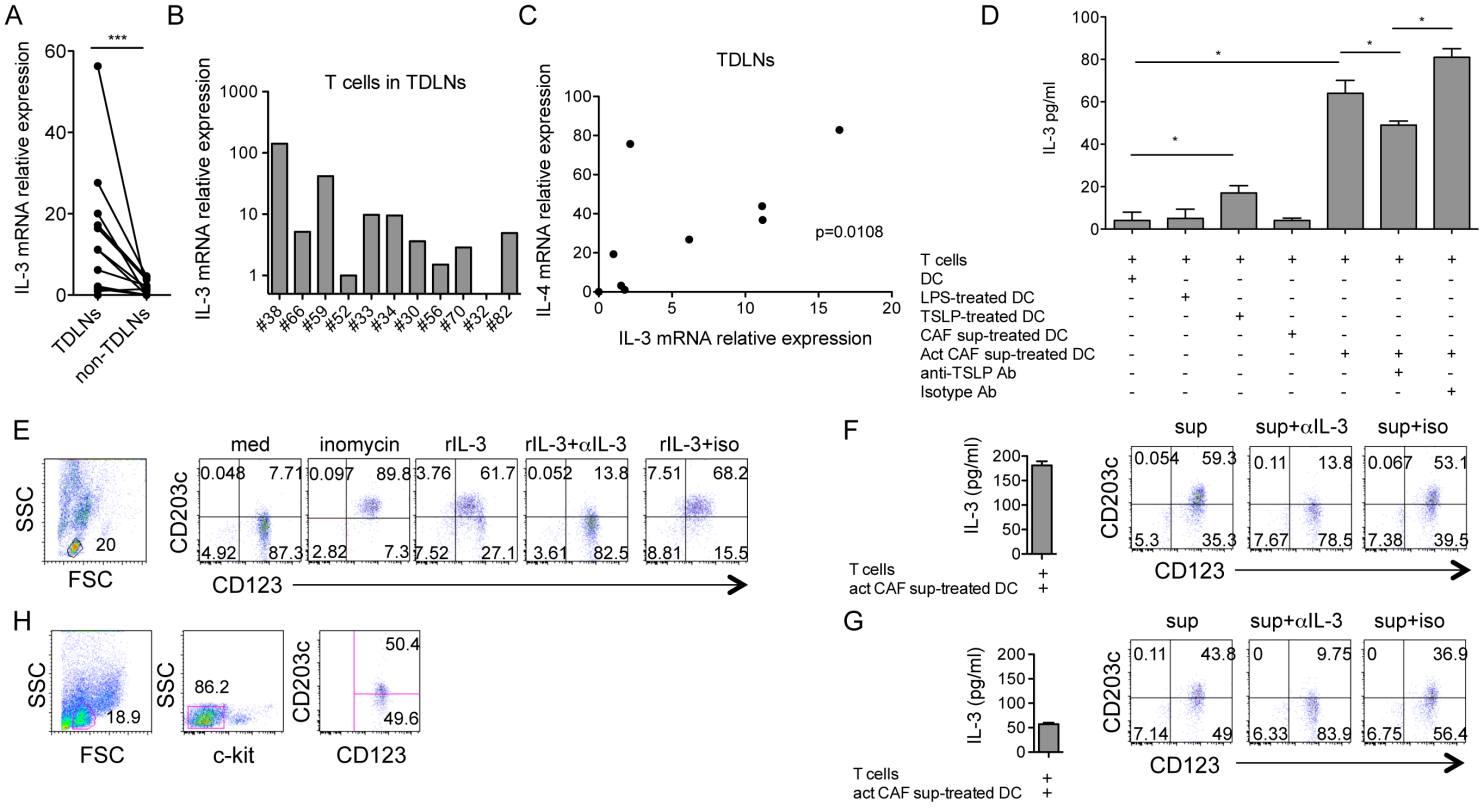


Figure 4

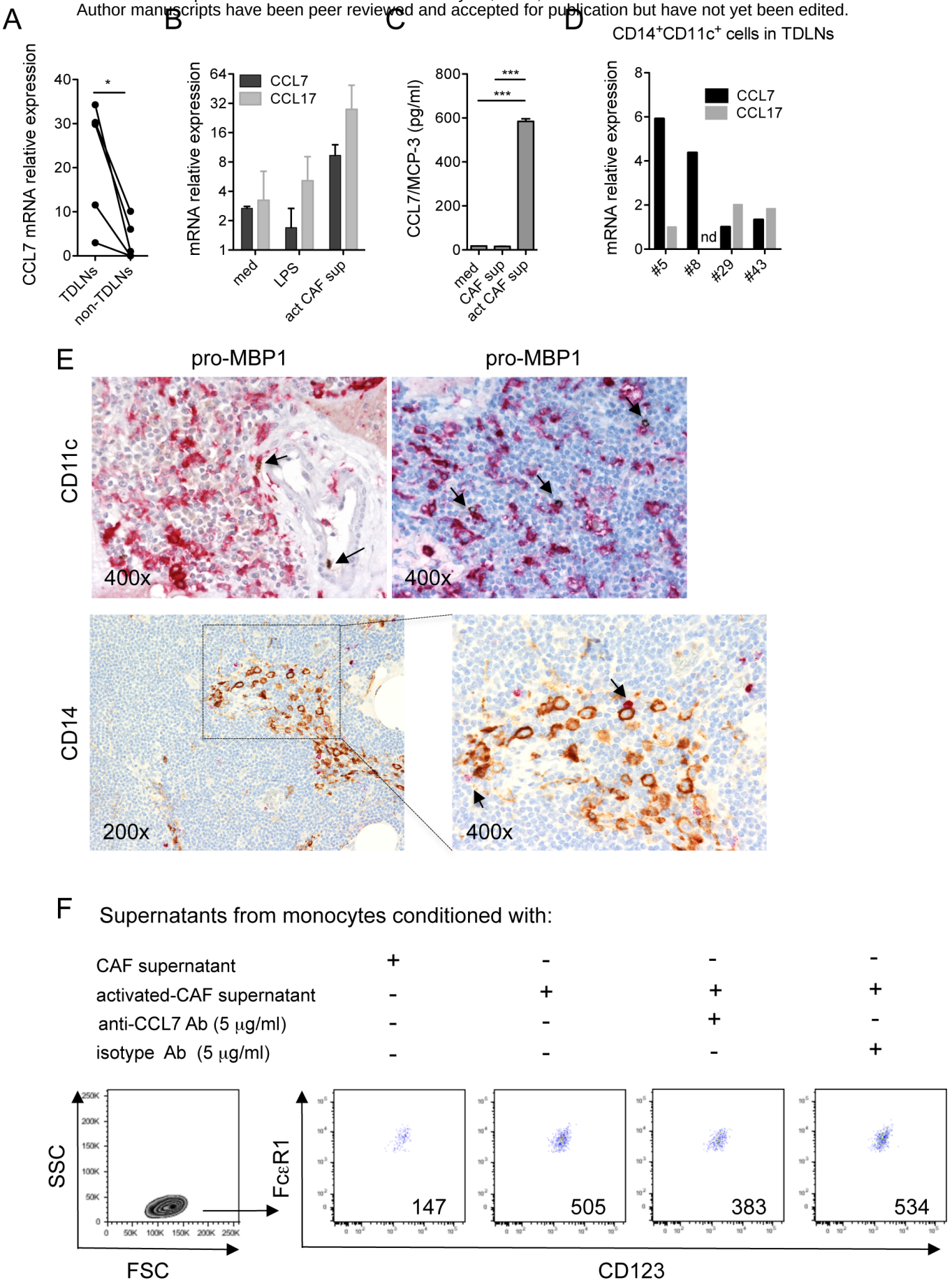
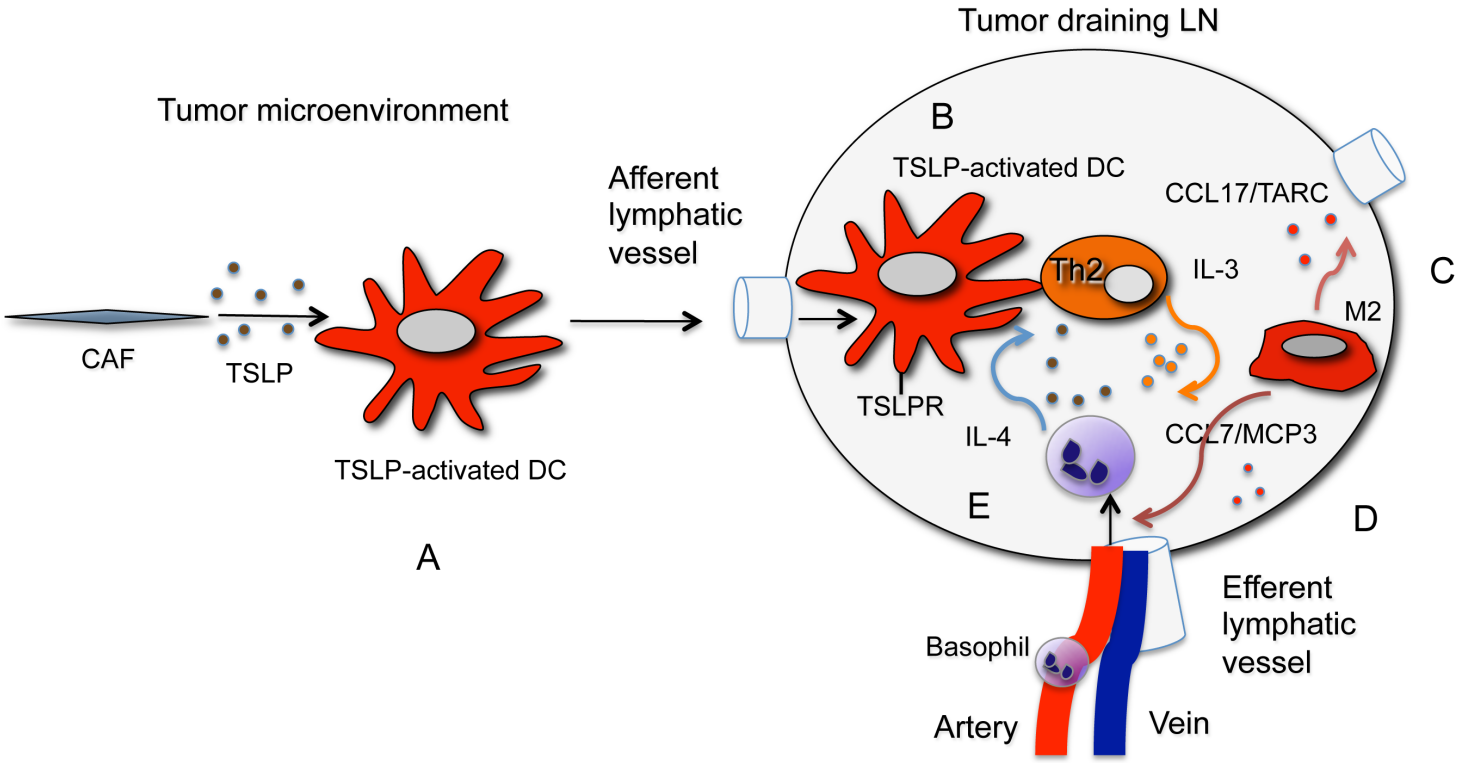


Figure 5



Revised Figure 6

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Basophil recruitment into tumor draining lymph nodes correlates with Th2 inflammation and reduced survival in pancreatic cancer patients

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