Tumor promotion by intratumoral plasmacytoid dendritic cells is reversed by TLR7 ligand treatment

Isabelle Le Mercier, Dominique Poujol, Amélie Sanlaville, Vanja Sisirak, Michael Gobert, Isabelle Durand, Bertrand Dubois, Isabelle Treilleux, Jacqueline Marvel, Jaromir Vlach, Jean-Yves Blay, Nathalie Bendriss-Vermare, Christophe Caux, Isabelle Puisieux, and Nadège Goutagny

1 Université de Lyon, Lyon, France; 2 Université Lyon 1, ISPB, Lyon, France; 3 Cancer Research Cancer of Lyon, INSERM U1052-CNRS UMR5286, Lyon, France; 4 Centre Léon Bérard, Lyon, France; 5 INSERM, U851, 21 avenue Tony Garnier, Lyon, France; 6 Université Lyon1, UMS3444 / US8, France; 7 Merck Serono International SA, Geneva, Switzerland; 8 LabEx DEVweCAN. # Authors equally contributed to the work.

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Corresponding Author: Dr Nadège Goutagny, Centre de Recherche en Cancérologie de Lyon, 28 rue Laennec, 69373 Lyon, France; Tel: (33) 4 78 78 29 64; Fax: (33) 4 78 78 27 20; Email: nadege.goutagny@lyon.unicancer.fr

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ABSTRACT

Plasmacytoid dendritic cells (pDC) are key regulators of antiviral immunity. In previous studies, we reported that pDC infiltrating human primary breast tumors represent an independent prognostic factor associated with poor outcome. In order to understand this negative impact of tumor-associated pDC (TApDC) we developed an orthotopic murine mammary tumor model that closely mimics the human pathology, including pDC and regulatory T cell (Treg) infiltration. We showed that TApDC are mostly immature and maintain their ability to internalize antigens in vivo and to activate CD4+ T cells. Most importantly, TApDC were specifically altered for cytokine production in response to toll-like receptor (TLR)-9 ligands in vitro while preserving unaltered response to TLR7 ligands (TLR7L). In vivo pDC depletion delayed tumor growth showing that TApDC provide an immune-subversive environment most likely through Treg activation thus favoring tumor progression. However, in vivo intratumoral administration of TLR7L led to TApDC activation and displayed a potent curative effect. pDC depletion and type I IFN neutralization prevented TLR7L antitumoral effect. Our results establish a direct contribution of TApDC to primary breast tumor progression and rationalize the application of TLR7 ligands to restore TApDC activation in breast cancer.
INTRODUCTION

Despite active immunosurveillance, some tumors still progress and escape through immunosubversion processes (1, 2). The understanding of the paradoxical role of the immune system during cancer development is a major challenge for new immunotherapy strategies.

Dendritic cells (DC), the most powerful antigen presenting cells (APC), play a key role in orchestrating adaptive immune responses. Most cancers, including breast tumors, are highly infiltrated by DC. Two main populations of DC, namely myeloid DC (mDC) and plasmacytoid DC (pDC), are found in mouse and human tissues. Functional alteration of mDC by the tumor microenvironment has been described as a mechanism to escape immunosurveillance. However, there is increasing evidences implicating also pDC in tumor immunity (3, 4).

pDC are key regulators of antiviral immunity at the interface of innate and adaptive immunity (5). pDC secrete rapidly large amounts of type I interferons (IFN), inflammatory cytokines, and chemokines in response to microbial and self-RNA or DNA recognized by endosomal Toll-like receptor (TLR)-7 and TLR9 respectively (6-8). After their encounter with viruses, pDC differentiate into mature DC and present viral antigens directing T cell responses with considerable flexibility (9). Uncontrolled production of type I IFN by chronically activated pDC contributes to autoimmune diseases (10). In contrast to immune activation, pDC were also shown to suppress or limit inflammatory responses to allo-Ag, allergens or oral Ag (11-14). In human breast cancer, we previously reported that pDC infiltrating the primary tumor represent an independent prognostic factor associated with poor outcome (15). pDC infiltrate other solid tumors with various consequences on immune response (16-18). We and others reported that tumor-associated (TA)pDC are altered for IFNα production (17, 19, 20) and favor Treg expansion via ICOS-ICOSL interaction (20, 20) that may contribute to tumor progression and explain their negative impact on patient survival (15). In contrast, TApDC
were shown to become efficient therapeutic targets after recruitment and activation by TLR7L in skin cancers (3, 21).

Depending on the context, TApDC could thus have negative or positive impact on anti-tumor immune responses. The current study in orthotopic mouse mammary tumor model was designed to understand whether TApDC directly contribute to breast tumor progression and whether they could be mobilized to favor tumor regression. We showed that NEU15 mammary tumor cell line implanted in immunocompetent mice are highly infiltrated by both TApDC and TATreg. Importantly, TApDC are functionally altered in their response to TLR9L and their in vivo depletion delay tumor growth. However, TApDC can be activated in vivo via TLR7L to induce tumor regression through a type I IFN-mediated mechanism. This study demonstrates a direct contribution of TApDC to breast tumor progression and identifies TLR7 ligands as new therapeutic strategies in breast cancer.
MATERIAL AND METHODS

Mice. Wild-type FVB/N and C57BL/6 (Charles River Laboratory, L’Arbresle, France), homozygous or heterozygous (named MMTV-Neu F1) FVB/N-MMTVneu-202Mul transgenic female mice ((22), Jackson Laboratory, USA) were used at 6-8 weeks of age. Mice were maintained in pathogen-free animal facility “AniCan” at the CRCL. Experiments were performed in accordance with the European and French laws and were validated by the local animal ethical evaluation committee (CECCAPP, Lyon, France).

Tumor Cell line and reagents. The NEU15 cell line was established from a spontaneous mammary tumor harvested from a MMTVneu transgenic female mouse. NEU15 cells were grown in vitro with 5% CO2 in DMEM (Life Technologies, France) supplemented with 10% heat inactivated fetal calf serum (PAA laboratories, France), 100U/ml penicillin, 100µg/ml streptomycin and 1% L-glutamine (Sigma-Aldrich, France).

TLR7 ligands were formaldehyde-inactivated influenza virus (A/Wisconsin/67/05) (1000 HAU/mL) (Gift from Sanofi Pasteur), CL075 (3µg/mL) and R848 (5µg/mL) (Cayla SAS, France), and SM360320 (5µg/mL, Janssen Infectious Diseases-Diagnostics BVBA, Belgium (23)). TLR9 ligands were CpG-A/ODN-2336 and CpG-B/ODN-1826 (5µg/mL, Cayla SAS).

In vivo transplanted orthotopic mammary tumor models. WT FVB or MMTV-neu F1 mice were injected with 5 x 10⁶ NEU15 cells into the fourth mammary fat pad. Tumor volume was calculated by using the ellipsoidal formula, \( \pi/6 \times \text{length} \times \text{width}^2 \). Tumor-bearing mice were euthanized at the experimental endpoint (volume > 2000mm³). TLR7L administration was performed as intratumoral or subcutaneous contralateral injection (50µl) as indicated. In pDC depletion experiment, mice were injected intraperitoneally with ascite-derived mab927 (200ul ½ diluted ascite, (24)) or purified 120G8 antibody (150µg/injection (25), BioXcell,
USA). Purified total IgG (Sigma-Aldrich) or rat IgG1 (BioXcell) were used as control. Anti-mouse IFNAR1 and rat IgG1 (50 μg i.t.) were from eBioscience (France).

**Enzymatic digestion and DC purification.** After enzymatic digestion (30 min at 37°C, type IA collagenase (1mg/mL) and DNase (0.1 mg/mL), red blood cell were lysed with Pharmlyse Buffer (BD Biosciences). For cell sorting, DC were enriched by anti-CD11c microbeads (Miltenyi Biotec), then stained with anti-CD11c-PE-Cy5, B220-PE and CD11b-FITC antibodies in presence of Fc Block. pDC were sorted as CD11c⁺CD11b⁺B220⁻ cells among live cells on a FACS Vantage sorter (BD Biosciences). Purity was routinely above 98%. For infiltrate analysis, CD45+ cells were first enriched from tumor single cell suspension by magnetic selection (Miltenyi Biotec) as detailed in supplementary Material and Methods.

**Cytokine secretion assays.** pDC were cultured at 0.25 x 10⁶ cells/ml with TLR-7 and -9 ligands in RPMI medium supplemented with 10% fetal calf serum, penicillin/streptomycin, L-glutamine, non essential amino-acids and 20ng/mL of human recombinant Flt3-ligand (PeproTech, USA). Supernatants were collected after 40h and assessed for cytokine production. RANTES (R&D systems, USA) and IFNα (PBL, USA) levels were measured by specific ELISA. IL-6 and MIP-1α were measured by Luminex multiplex bead cytokine assay (MILLIPLEX Mouse Cytokine / Chemokine kit, Millipore).

**Mixed lymphocyte reaction.** Allogeneic CD4⁺ T cells were purified using anti-CD4 coated microbeads (Miltenyi Biotec) from naïve spleen of C57BL/6 mice depleted of Gr1, MHC II, CD11b, and CD8-expressing cells. CD4⁺ T cells (5 x 10⁴ per well) were cultured in triplicate for 5 days with pDC using indicated TLR ligands. At day 4, half of the medium was collected for IFN-γ determination by ELISA (R&D systems). T cell proliferation was assessed by [³H]-
thymidine incorporation during the last 18 hours (0.5µCi/well, GE healthcare, USA.). \[^{3}H\]-thymidine incorporation was measured as counts per minute (cpm) by liquid scintillation counting (MicroBeta; PerkinElmer, USA).

**Statistical analysis.** Statistical analyses were performed using Prism 5 software. Differences between groups were analyzed using the Mann-Whitney test for non-parametric and unpaired samples. Gehan Breslow Wilcoxon test was used to compare survival curves. P values less than 0.05 were considered significant (* p<0.05, ** p<0.01, *** p<0.001).
RESULTS

Transplantable HER2/neu-expressing tumors escape immune response in WT mice while preserving HER2/neu expression. In order to characterize the potential role of pDC in primary breast tumor progression, we selected a clinically relevant murine mammary tumor model. HER-2/neu FVB/N transgenic mice express the rat proto-oncogene her2/neu under the control of the mammary specific MMTV promoter. Those mice develop focal, poorly differentiated ER\(^+\)/neu\(^+\) spontaneous mammary carcinomas (Figure S1A/B). Due to long and variable latency of tumor appearance, a stable HER-2/neu-expressing cell line (referred to as NEU15) was derived in vitro from a spontaneous tumor. NEU15 was then transplanted by orthotopic injection into syngeneic mice. Transgenic mice tolerated rat HER2/neu as a self-protein as evidenced by the absence of specific immune responses due to central tolerance mechanisms and the aggressive tumorigenicity of NEU15 tumor in MMTV-neu F1 mice (named NEU15F1, Figure 1A). In contrast, rat HER2/neu was perceived as a foreign antigen by the immune system of wild type (WT) mice, as evidenced by the induction of high levels of anti-HER2/neu antibody of IgG1 isotype that resulted in delayed NEU15 tumor (named NEU15WT) growth (Figure 1A/B). Accordingly, NEU15WT tumor-bearing mice displayed (data not shown). Despite immune pressure, NEU15WT tumors maintained similar HER2/neu expression to that of NEU15F1 tumors (Figure 1C). The resistance of NEU15WT tumor cells to immunoediting implies that sustained HER2/neu expression is essential for NEU15 tumor cell survival and suggests an immunosubversion by the tumor microenvironment.

NEU15WT tumors are highly infiltrated by pDC and Treg. While leukocytes are found in all tumors, NEU15WT tumors appeared more infiltrated by CD45\(^+\) cells (22.9 ± 7,3%) than NEU15F1 tumors (8.9 ± 5.3%) (Figure S2A).
A thorough analysis of immune cell infiltrate was performed by multiparametric flow cytometry on NEU15WT tumor single cell suspension (Figure 2A). T cells (CD3⁺) and NK cells (NKp46⁺) represented about 10 to 15% of leukocytes, while B (CD19⁺) represented a minor part of the infiltrate (Figure 2B). Macrophages (CD11b⁺MHCIIintLy6G⁻/C⁻), monocytes (CD11b⁺MHCIIintLy6C⁺) and neutrophils (CD11b⁻CD11cintLy6G⁻) infiltration (also described as MDSC) represented a moderate part of the immune infiltrate, in contrast to more aggressive mammary tumor models (4T1 and TS/A). Interestingly, SiglecF⁺ myeloid cells (most likely eosinophils or SiglecF⁺ macrophages) represented almost 20% of the infiltrate. Remarkably, DC represented the most important infiltrating population in all tumors thus identifying this model as particularly relevant for TADC functional characterization. CD11b⁺ DC (CD11c⁺CD11b⁺MHCIIhi) represented the major part (about 20% of leukocytes) when compared to CD8α⁺ DC (CD11c⁻CD8α⁻SiglecH⁻) and pDC (CD11c⁻ SiglecH⁺) (Figure 2B).

Frequency of most immune cells was similar in both tumor types (data not shown) except for pDC and Treg that were more abundant in NEU15WT than NEU15F1 tumors (Figure 2C/D, and S2B). Finally, histological analyses confirmed that both TApDC and TATreg are found within the tumor mass, with pDC mostly localized in the tumor bed and Treg in both tumor bed and immune infiltrate areas (Figure 2E).

Taken together, these results show that NEU15WT tumor represents an interesting immunosubversion model closely mimicking our observations in human breast cancer with increased pDC and Treg recruitment possibly contributing to escape to immunosurveillance (15, 20, 20, 26).

**Tumor-associated pDC are functionally immature and can mediate CD4⁺ T cell activation.** TApDC were gated based on their high expression of CD11c and Siglec-H
They expressed high levels of BST2, B220 and Ly6C while lacking Ly6G and CD11b thus confirming their identity (Figure 3A). Moreover, they showed heterogeneous CD8α expression. TApDC were immature with no surface expression of CD40, CD80 and CD86, and intermediate levels of MHC-II (Figure 3A). This phenotype resembled to the one of pDC found in naïve spleen (data not shown). Furthermore, in vivo phagocytic activity was weak but similar to spleen-derived pDC (Figure S3) suggesting that immature TApDC may uptake tumor Ags.

pDC were sorted from tumor or spleen and cultured with allogeneic naïve CD4+ T cells in the presence or not of various TLR ligands. Regardless of their tissue of origin, freshly isolated pDC did not activate CD4+ T cells (Figure 3B and C). However, pDC maturation through TLR7 ligands, and to less extent TLR9 ligands, induced effector T cell proliferation (Figure 3B) as well as IFNγ production (mean IFNγ (pg/ml) for spleen vs tumor pDC respectively: Ctrl, 30 vs 6; TLR7 ligands, 764-2312 vs 415-2275; TLR9 ligands, 84-278 vs 55-88 pg/ml) (Figure 3C). In conclusion, TApDC are phenotypically and functionally immature and may acquire abilities to activate anti-tumor effector T cells upon TLRL activation, as spleen-derived pDC.

TApDC exhibit an abrogated cytokine response to TLR9 but not TLR7 ligands. As the capacity of APC-derived cytokines is crucial to shape the immune response, we measured the ability of TApDC to secrete cytokines after in vitro TLR stimulation. Interestingly, IFNα production by TApDC in response to CpG-A was strongly inhibited, with 30-fold less IFNα than in naïve spleen pDC (294.4 ± 295.9 vs 10.65 ± 27.1) (Figure 4A). This alteration was confirmed using CpG-B/ODN-1826 with a 5-fold decrease in IFNα production (Figure 4A). In contrast, TApDC were as potent as naïve spleen pDC to produce IFNα in response to Flu.
Other TLR7 ligands, such as CL075 or SM360320 (23), two synthetic TLR7 ligands, did not trigger significant IFNα even in spleen pDC.

Similarly the production of inflammatory cytokines by TApDC was specifically altered in response to CpG-A/B whereas responses to Flu, CL075 and SM360320 remained mostly unchanged (Figure 4B). In particular, production of MIP-1α (323.7 ± 261.8 vs 83.4 ± 144.1 for CpG-A) and IL-6 (67.8 ± 60.9 vs 11.9 ± 17.5 for CpG-A; 1,999.8 ± 292.7 vs 292.7± 344.6 for CpG-B) by TApDC in response to CpG-A and/or CpG-B were significantly reduced when compared to naive spleen pDC.

We then assessed a role for TLR9 downregulation in this alteration. Both TLR9 and TLR7 mRNA expression were slightly but similarly reduced in tumor versus spleen-derived pDC (Figure S4). Furthermore, production of cytokine such as TNFα was not altered in tumor versus spleen-derived pDC in response to TLR9L (data not shown). Altogether, this shows that TLR9 receptor downregulation cannot merely explain the specific alteration of cytokine production in response to TLR9L when compared to TLR7L.

**pDC depletion delays tumor growth in vivo.** To determine whether such TApDC contributes to tumor growth, pDC were depleted in vivo using anti-BST2 depleting mAbs (24, 25). WT mice were treated every other day by i.p. injection from the day prior to tumor implantation until the experimental endpoint. A significant decrease of the tumor volume was observed upon pDC depletion from day 14 post-implantation (Figure 5A). Tumor growth was followed over time and mice were euthanized when tumor size reached the endpoint. Survival curve analysis showed an increase in median survival times from 35 to 43 days (Figure 5B). Specific and effective pDC depletion in the tumor upward of 80% was validated by flow cytometry (Figure 5D) and functional IFNα response to TLR7-L intratumoral injection at the endpoint (Figure 5C). Those data show that effective pDC depletion in the tumor...
microenvironment delays tumor growth and increases mice survival. These results are in concordance with our observation in human breast cancer demonstrating that recruitment of pDC within the tumor directly contributes to poor clinical outcome (15, 27).

**TLR7 triggering induces tumor regression in vivo.** As breast TApDC respond to TLR7L *in vitro*, we next assessed the possibility to activate TApDC *via* SM360320, a TLR7 agonist shown to be *in vivo* a robust IFNα inducer and a potent adjuvant (23), in order to revert their tumor-promoting ability. Intratumoral injections of TLR7-L (50μg) lead to potent tumor regression when compared to vehicle-treated mice (**Figure 6A**). TLR7L treatment induced a strong increase in complete response with 90% in TLR7L-treated group versus 30% of spontaneous regression in the vehicle-treated group (p=0.0198, data not shown). Importantly, 100% of cured mice were protected against a subsequent orthotopic contralateral challenge of NEU15 cells (3 months later) (data not shown). In contrast to i.t. injection, contralateral subcutaneous injection of TLR7L did not induce significant tumor regression (**Figure 6B**). Both i.t. and contralateral subcutaneous injections led however to similar range of plasmatic IFNα levels suggesting that i.t. route is necessary to mobilize anti-tumor activity of TApDC (**Figure 6C**).

Analyses of intratumoral immune infiltrates exhibited an increase in leukocyte frequency as soon as 24h post TLR7L injection (**Figure S6A**). While no major changes in the frequency of most immune cells could be noticed (data not shown), a significant and specific increase in monocyte infiltration was observed (**Figure S6B**). As pDC are the most likely target of such TLR7L *in vivo*, we explored whether i.t. TLR7L injection affected pDC frequency and function *in vivo*. While pDC frequency remained unchanged (data not shown), TLR7L induced strong increase in MHC-II expression and costimulatory molecules at their surface (**Figure 6D**). In contrast, CpG did not activate TApDC thus confirming our *in vitro* data.
Specific activation of TApDC was confirmed, as neither TA-CD8α+ DC nor TA-CD11b+ DC displayed increase in MHC-II (nor CD80, CD86, data not shown) expression upon TLR7 triggering (Figure 6D).

Finally, changes in gene expression were analyzed in NEU15WT tumors 8 hours post i.t. injection of TLR7L by TaqMan® Low density array (TLDA) mouse immune assay (Supplementary Material and Methods). Data are presented as fold changed over the non-treated conditions and displayed as a Heat Map (Figure 6E) and detailed in Figure S5. Genes that displayed fold changes higher than 10 were selected to highlight the most significant changes in gene expression upon TLR7 i.t. injection. These data depicted an increased infiltration in immune cells such as myeloid cells (H2-Ea-ps, Nos2) and in particular cytotoxic T cells (Cd3e, Cd8, Tbx21), via chemokine-mediated recruitment (Cxcl11, 2413 ± 3409 fold changes; Ccr7, 58 ± 53 fold changes). The increase in perforin and granzyme genes (125 ± 147 and 185 ± 209 fold changes respectively) also indicated a potent cytotoxic response. A Th1-type T cell response was seen via the sharp increase in genes related to APC maturation (Cd28, Cd40, IL12b), IFNγ(123 ± 151 fold changes) and IFNγ-induced genes (Cxcl10, Ccl5, Stat1) production. Interestingly, the induction of this genes, with the exception of IL10, was type I IFN-dependent as gene increase was no longer seen when anti-IFNAR1 antibody was co-injected with TLR7L (Figure S5A). In parallel, an increase in type I IFN genes (IFNa and Mx1) was seen in samples treated by TLR7L by quantitative PCR (Figure S5B).

Altogether these data demonstrated the induction of type I IFN response as well as Th1 cytotoxic T cells response leading to tumor regression.

In contrast to CpG, in vivo TLR7 antitumoral activity requires type I IFN production by pDC. In order to explore whether TLR7L antitumoral activity was mediated by pDC, pDC were depleted the two days prior to TLRL injection. As previously observed with a dose of
50µg, 10µg of TLR7L also lead to a significant decrease of tumor volume (day35) (Figure 7A). pDC depletion in such short-term schedule led to a delay in tumor growth as previously observed (Figure 7A). Finally, pDC depletion completely abrogated TLR7L-mediated antitumoral effect (1046 ± 221.58 mm³ for w/o pDC vs 1266.5 ± 335.67 mm³ for TLR7L w/o pDC), demonstrating that pDC mediate the antitumor effect of TLR7L in vivo (Figure 7C). The efficiency of pDC depletion was confirmed phenotypically (data not shown) and functionally at the type I IFN systemic plasmatic level (Figure 7B).

We then assessed whether inhibition of type IFN signaling affected TLR7L activity. For that matter, anti-IFNAR1 or control antibodies were co-administered i.t. with TLR7L (10µg). IFNAR inhibition resulted in a significant decrease of TLR7L anti-tumoral activity showing the requirement for type I IFN in TLR7L activity (752.1 ± 135.3 mm³ for TLR7L vs 1392.1 ± 181.8 mm³ for TLR7L w/o IFNAR). The efficacy of anti-IFNAR antibodies was confirmed by qPCR on tumor extracts with a decrease in IFN stimulated gene expression upon TLR7L treatment in the context of type I IFN blockade (data not shown).

As TApDC were shown to lack both in vivo and in vitro the ability to respond to CpG, the effect of this TLR9L on tumor growth was assessed. Unexpectedly, intratumoral administration of CpG-B induced tumor regression (771.7 ± 295.3 mm³ for CpG vs 1826.6 ± 196.8 mm³ for control). Interestingly, inhibition of tumor growth by CpG was delayed in comparison with TLR7L, which started to decrease the tumor volume as soon as after the first injection. However the mechanism seems different as intratumoral CpG injection did not lead to any detectable IFNα production in contrast with TLR7L (data not shown). Surprisingly, pDC depletion altered CpG antitumoral activity (Figure 7D), however inhibition of type I IFN signaling did not (Figure 7E). These results suggest that pDC indirectly affect CpG activity through a distinct type I IFN-independent mechanism.
Altogether, those data demonstrate that in contrast to CpG, TLR7L antitumoral activity is mediated by pDC and their ability to secrete large amount of type I IFN.
DISCUSSION

In line with the negative prognostic value of pDC infiltration in human breast tumors (15), we demonstrate for the first time *in vivo*, that mouse mammary TApDC favor primary tumor growth but can be activated *via* TLR7 triggering to mediate antitumoral response and subsequent tumor regression.

We developed a clinically relevant tumor model in which the HER2/neu+ NEU15 cell line developed in WT hosts escapes from immunosurveillance through pDC and Treg-mediated immunosubversion, thus closely mimicking our observations in human breast cancer (15, 20, 20, 26).

First, we showed *in vitro* and *in vivo* that TApDC from NEU15WT tumors were specifically altered in their ability to respond to TLR9 but not TLR7 ligands. These data are consistent with the specific alteration of TLR9 response previously reported by us (19, 20) and others on both human tumor and immune cells (17, 28). While pDC hyporesponsiveness to TLR9 ligands might be explained by receptor downregulation (17, 28), we demonstrated that it is rather due to a specific interference of the tumor microenvironment, for instance *via* IRF7 downregulation (29). Tumor-induced TLR9 loss of function might represent an immunosubversion mechanism in order to avoid immune alert by putative endogenous ligands for TLR9 within the tumor microenvironment, such as described in autoimmune disorders (30-32).

Second, we demonstrated for the first time in primary breast tumors that TApDC favored breast tumor progression. *In vivo* depletion of pDC indeed significantly slowed down tumor growth. While antibody-mediated pDC depletion is quite efficient, variation in its efficacy might account for discrepancies in the beneficial effect of pDC depletion in short-term depletion settings (Figure 7B and D). The use of a pDC-deficient mouse model (33) will formerly demonstrate the extent of pDC role in tumor progression. To our knowledge this is a
unique proof of a direct role of pDC in favoring primary tumor growth in solid tumors. pDC were previously shown to regulate growth of multiple myeloma (MM) cells (34) and more recently to favor bone metastasis of breast cancer cells (35).

Accumulating evidences have shown a specialized role of pDC in the induction of peripheral tolerance (11-14, 36) through Ag-specific T cell deletion (12) or differentiation/expansion of suppressive T cells (11, 14, 31, 37-44). NEU15WT tumors were indeed more infiltrated by Treg. For instance, we observed in preliminary experiments a decrease in TATreg infiltration after pDC depletion. This role of pDC would be consistent with our recent reports in human breast cancer, showing that IFNα deficient TApDC from human breast tumors favor Treg expansion via ICOS-ICOSL interaction (20, 27) that may contribute to tumor progression and explain their negative impact on patient survival (15). Whether tumor pDC are able to modulate Treg function/differentiation remains to be addressed.

Despite their negative impact on tumor progression, TApDC could be activated in vivo via intra-tumoral injection of TLR7L, thus mediating a Th1 signature, as evidenced by TLDA analysis, and subsequent tumor regression. TLR7L induced long term protective memory response as 100% of cured mice were protected against subsequent tumor challenge. In vivo depletion of pDC abrogated the therapeutic activity of TLR7L, demonstrating the central role for TApDC in TLR7L-mediated antitumor response. Importantly, we demonstrated that this therapeutic activity is mediated by locally-induced and not systemic type I IFN. This points towards the importance of intratumoral TApDC activation leading to type I IFN production and subsequent additional activities (cross-presentation, Treg neutralization). We indeed observed that type I IFN neutralization led to the inhibition of the intratumoral Th1 signature. The antitumor activity of Imiquimod, another TLR7L, is dependent upon CD8α+ pDC that harbor direct tumor killing activity mediated by granzyme B and/or TRAIL leading to subsequent capture and antigen cross-presentation (3, 21).
However we did not observe any increase in TRAIL expression on our TApDC upon TLR7L treatment (data not shown) suggesting a different mechanism of action. However, considering our results in human models that type I IFN led to the inhibition of TATreg proliferation (20), the potential role of Treg inhibition in this TLR7L antitumoral activity will be carefully assessed.

While we demonstrated both in vitro and in vivo that CpG could not activate TApDC, this TLR9L unexpectedly induced tumor regression. While is antitumoral activity was reduced upon pDC depletion, IFNAR inhibition did not affect their therapeutic action showing a different mechanism of action than TLR7L. We hypothesize that pDC depletion somehow impact on key effectors of CpG-mediated antitumoral response. We indeed observed an increase in macrophage infiltration upon CpG treatment (Figure S6B) that was reduced upon pDC depletion (data not shown). While tumor-associated macrophages (TAM) are commonly associated with tumor development and progression, antitumor activity can be achieved by targeting TAM recruitment and polarization toward M1 phenotype, a process to which CpG was shown to participate (45, 46). CpG-mediated antitumoral mechanisms will need to be further clarified.

Based on promising results of the literature, ongoing phase I/II clinical trials are currently evaluating the therapeutic potential of TLR agonists for the treatment of various type of cancer (47). In the light of our observation that TApDC remain responsive to TLR7L while lacking response to CpG ODN, the therapeutic potential of TLR7 agonists in human breast tumors shall be considered.

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FIGURE LEGENDS

Figure 1: NEU15WT tumors escape immunosurveillance without immunoediting. (A) NEU15 tumor cells derived from a MMTVneu spontaneous tumor were injected into WT or MMTVneu F1 female mice. Data represent the mean ± SEM of the calculated tumor volume for five individual mice. (B) Concentrations of anti-Neu antibodies in sera from MMTVneu mice bearing NEU15WT or NEU15F1 tumors were measured. (C) Representative immunohistochemical staining on paraffin-embedded sections of NEU15WT and NEU15F1 tumors using HPS stain or anti-HER2/Neu antibody as indicated. Magnification X200. TUM, tumor bed.

Figure 2: Increased TApDC and regulatory T cell infiltration in NEU15WT tumors escaping immunosurveillance. (A, C) NEU15WT tumors were analyzed for immune infiltration according to the gating strategy presented in (A). (B) Percentage of immune cells were evaluated among leukocytes from NEU15WT tumor single cell suspensions. (C-D) Percentages of tumor-infiltrating pDC and Treg cells (gating strategy detailed in Figure S3A/B) were evaluated in NEU15WT and NEU15F1 tumors. (E) Representative immunohistochemical staining on paraffin-embedded sections of NEU15WT tumors using anti-BST2 or -Foxp3 antibodies. Magnification X200. SiglecF+ m.c., myeloid cells; Neutro., neutrophil; Mφ, macrophage; Mono., monocyte.

Figure 3: NEU15WT TApDC are phenotypically and functionally immature. Freshly isolated TApDC were characterized in NEU15WT tumors. (A) Representative expression profile of surface molecules on gated TApDC are shown as overlay of specific (gray) and control (white) stainings. (B, C) Resting and activated TApDC or naive WT spleen pDC were
incubated with allogeneic CD4+ T cells. T cell proliferation (B) and IFN-γ production (C) were determined. Data are expressed as mean ± SEM of 7/8 experiments. Statistical analyses of TLRL-treated versus medium ((−)) condition are shown for spleen and tumors.

**Figure 4: NEU15WT TApDC exhibit an abrogated cytokine response to TLR9 but not TLR7 ligands.** TApDC and naive WT spleen pDC were incubated with indicated TLRL. Supernatants were collected and analyzed for IFN-α (A), MIP-1α and IL-6 (B) production. Data represent the mean ± SD from 3 to 12 independent samples.

**Figure 5: pDC depletion delays tumor growth in vivo.** (A-C) WT mice (n=20 per group) were treated with purified pDC-depleting 120G8 or rat IgG1 control antibody from one day prior to tumor implantation and every other day until tumors reached the experimental endpoint. (A) Data represent the mean tumor volume mean ± SEM for twenty mice. (B) Kaplan-Meier survival plot of mice stratified in those with pDC depletion versus none. Data are from one representative out of 3 independent experiments. (C-D) In vivo depletion of pDC was analyzed functionally (C) and phenotypically by flow cytometry in tumors (D). (C) TLR7L (10µg) was administrated by i.t. injection at experimental endpoint and serum were harvested 3 hours after for IFNα levels measurement (rat IgG1 control ●, pDC-depleted ○). (D) Dot plot representation of CD11b/SiglecF staining gated on CD45+CD11c+lin− cells after dead cells and doublet exclusion for one representative mouse of each group.

**Figure 6: Intratumoral TLR7L injection induces tumor regression.** Mice were treated with 50µg SM360320 TLR7L at day 13 and 20 post NEU15 tumor implantation by intratumoral (A-E) or subcutaneous contralateral (B, C) injections. Controls were injected with vehicle only (A, B) Data represent the mean tumor volume ± SEM for ten mice. (C)
Serum was harvested 3 hours after TLR7L injection for IFNα levels measurement. (D) Intratumoral DC activation was assessed 24 hours post-intratumoral TLRL injection by flow cytometry. Representative expression profiles of costimulatory molecules on gated TApDC, CD8α+ or CD11b+ DC are shown. (E) Fold changes in gene expression upon intratumoral TLRL injection. TLDA mouse immune array were performed on tumor samples from control and TLRL treated mice. Data were calculated as fold changes over non-treated control condition and presented as Heat map. Genes with fold changes over 10 are listed here.

Figure 7: Therapeutic activity of TLR7L depends on TApDC and type I IFN production. NEU15WT-bearing mice were treated with 10µg TLR7L (A-C) or CpG-B (D-E) at day 13 and 20 (n=10-15/group). (A, D) pDC were depleted from WT mice the two days prior to TLRL injection. (B) Serum were harvested 3 hours after TLRL injection for IFNα levels measurement. (C, E) Mouse IFNAR1 or rat IgG1 control antibodies were co-injected with TLRL. (A, C-E) Data represent the mean tumor volume ± SEM.
REFERENCES


(38) Bilsborough J, George TC, Norment A, Viney JL. Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. Immunology 2003;108:481-92.
Figure 1

A

Tumor volume (mm$^3$)

- Neu15F1
- Neu15WT

days post-implantation

B

ant-Neu Aβ (μg/ml)

NEU15WT NEU15F1

C

NEU15WT NEU15F1

HPS

TUM

Her2/neu

Mammary Gland

Mammary Gland
Figure 2
Figure 4

**A**

[Bar chart showing IFN-γ (ng/mL) levels for different treatments: Flu, CL075, SM360320, CpG-B, CpG-A for TLR7-L and TLR9-L. Significance marked with *.

**B**

[Bar charts for CCL3 and IL-6 (pg/mL) concentrations in Spleen and Tumor for TLR7-L and TLR9-L. Significance marked with *.

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 5
Figure 6
Tumor promotion by intratumoral plasmacytoid dendritic cells is reversed by TLR7 ligand treatment

Isabelle Le Mercier, Dominique Poujol, Amelien Sanlaville, et al.

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