Tumor Promotion by Intratumoral Plasmacytoid Dendritic Cells Is Reversed by TLR7 Ligand Treatment

Isabelle Le Mercier1,2,3, Dominique Poujol1,2,3,4, Amélien Sanlaville1,2,3, Vanja Sisirak1,2,3, Michael Gobert1,2,3, Isabelle Durand1,2,3,4, Bertrand Dubois5,6, Isabelle Treilleux4, Jacqueline Marvel5,6, Jaromir Vlach8, Jean-Yves Blay1,2,3,4,7, Nathalie Bendriss-Vermare1,2,3,4, Christophe Caux1,2,3,4,7, Isabelle Puisieux1,2,3,4, and Nadège Goutagny1,2,3,4,7

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. 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 pDC depletion delayed tumor growth, showing that TApDC provide an immunosubversive 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. Depletion of pDC 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. Cancer Res; 73(15); 1–12. ©2013 AACR.

Introduction

Despite active immunosurveillance, some tumors still progress and escape through immunosurveillance 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, 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 dendritic cell. Two main populations of dendritic cell, namely myeloid dendritic cell (mDC) and plasmacytoid dendritic cell (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 also increasing evidence implicating 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 IFNs, 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 dendritic cell 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 (TApDC are altered for IFN-α production (17, 19, 20) and favor regulatory T cell (Treg) expansion via ICOS–ICOSL interaction (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 antitumor immune responses. This study

Authors’ Affiliations: 1Université de Lyon, Lyon, France; 2Université Lyon 1, ISPB; 3Cancer Research Cancer of Lyon, INSERM U1052-CNRS UMRS282; 4Centre Léon Bérard; 5INSERM, U851; 6Université Lyon1, UMS3444/US8; and 7LabEx DEVeCan, Lyon, France; and 8Merck Serono International SA, Geneva, Switzerland

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

C. Caux, I. Puisieux, and N. Goutagny have equally contributed to this work.

Corresponding Author: Nadège Goutagny, Centre de Recherche en Cancérologie de Lyon, 28 rue Lannec, 69373 Lyon, France. Phone: 33-4-78-78-27-20; Fax: 33-4-78-78-29-64; E-mail: nadege.goutagny@lyon.unicancer.fr

doi: 10.1158/0008-5472.CAN-12-3058
©2013 American Association for Cancer Research.
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 delays tumor growth. However, TApDC can be activated in vivo via TLR7L to induce tumor regression through a type 1 IFN-mediated mechanism. This study shows a direct contribution of TApDC to breast tumor progression and identifies TLR7 ligands as new therapeutic strategies in breast cancer.

Materials and Methods

Mice

Wild-type FVB/N and C57BL/6 (Charles River Laboratory), homozygous or heterozygous (named MMTV-Neu F1) FVB/N-MMTVneu-202Mul transgenic female mice (Jackson Laboratory; ref. 22) were used at 6 to 8 weeks of age. Mice were maintained in pathogen-free animal facility "AniCan" at the Cancer Research Center of Lyon. Experiments were conducted in accordance with the European and French laws and were validated by the local animal ethical evaluation committee (Comité d’Équité Commun CLB Animal transit ENS PBES P4).

Tumor cell line and reagents

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

TLR7 ligands were formaldehyde-inactivated influenza virus (A/Wisconsin/67/05; 1,000 HAU/mL; gift from Sanofi Pasteur), Cl075 (3 μg/mL) and R848 (5 μg/mL; Cayla SAS), and SM360320 (5 μg/mL; Janssen Infectious Diseases-Diagnostics BVBA; ref. 23). TLR9 ligands were CpG-A/ODN-2336 and CpG-B/ODN-1826 (5 μg/mL; Cayla SAS).

In vivo transplanted orthotopic mammary tumor models

Wild-type (WT) FVB or MMTV-neu F1 mice were injected with 5 × 10^6 NEU15 cells into the fourth mammary fat pad. Tumor volume was calculated by using the ellipsoidal formula, \( V = \frac{4}{3} \pi a b c \), where \( a \) is the length, \( b \) is the width, and \( c \) is the thickness of the tumor. Tumor-bearing mice were euthanized at the experimental endpoint (volume > 2,000 mm³). TLR7L administration was conducted as intratumoral (i.t.) or subcutaneous contralateral injection (50 μL) as indicated. In pDC depletion experiment, mice were injected intraperitoneally with ascite-derived mab927 (200 μL half diluted ascite; ref. 24) or purified 120G8 antibody (150 μg/injection; BioXcell; ref. 25). Purified total IgG1 (Sigma-Aldrich) or rat IgG1 (BioXcell) were used as control. Anti-mouse IFNAR1 and rat IgG1 (50 μg) were from ebioscience.

Enzymatic digestion and dendritic cell purification

After enzymatic digestion [30 minutes at 37°C, type IA collagenase (1 mg/mL) and DNase (0.1 mg/mL)], red blood cells were lysed with Phamlyse Buffer (BD Biosciences). For cell sorting, dendritic cell were enriched by anti-CD11c microbeads (Miltenyi Biotec), then stained with anti-CD11c-PE-Cy5, B220-PE, and CD11b-ITC 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 Materials and Methods.

Cytokine secretion assays

pDC were cultured at 0.25 × 10^6 cells/mL with TLR7 and TLR9 ligands in RPMI medium supplemented with 10% fetal calf serum, penicillin/streptomycin, l-glutamine, nonessential amino acids, and 20 ng/mL of human recombinant Flt3-ligand (PeproTech). Supernatants were collected after 40 hours and assessed for cytokine production. CCL5 (R&D Systems) and IFN-α (PBL) levels were measured by specific ELISA. Interleukin (IL)-6 and CCL3 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 naive spleen of C57BL/6 mice depleted of Gr1, MHC II, CD11b, and CD8-expressing cells. CD4+ T cells (5 × 10^6 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 (0.5 μCi/well; GE Healthcare). [3H]-Thymidine incorporation was measured as counts per minute (cpm) by liquid scintillation counting (MicroBeta; Perkin-Elmer).

Statistical analysis

Statistical analyses were conducted using Prism 5 software. Differences between groups were analyzed using the Mann-Whitney test for nonparametric 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

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-negative breast cancers (Supplementary Fig. S1A and S1B). Because of 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; Fig. 1A). In contrast, rat HER2/neu was perceived as a foreign antigen by the immune system of 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; Fig. 1A and 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 (Fig. 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**

Although leukocytes are found in all tumors, NEU15WT tumors seemed more infiltrated by CD45^+^ cells (22.9 ± 7.3%) than NEU15F1 tumors (8.9 ± 5.3%; Supplementary Fig. S2A). A thorough analysis of immune cell infiltration was conducted by multiparametric flow cytometry on NEU15WT tumor single cell suspension (Fig. 2A). T cells (CD3^+^) and NK cells (NKp46^+^) represented about 10% to 15% of leukocytes, whereas B (CD19^+^) represented a minor part of the infiltrate (Fig. 2B). Macrophages (CD11b^-MHCIIm^low^Ly6G^+/C^-), monocytes (CD11b^-MHCIIm^low^Ly6C^+^) and neutrophils (CD11b^-CD11c^+/CD11e^low^Ly6G^+^) 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 orSiglecF^+^ macrophages) represented almost 20% of the infiltrate. Remarkably, dendritic cell represented the most important infiltrating population in all tumors thus identifying this model...
as particularly relevant for TADC functional characterization. CD11b⁺ dendritic cell (CD11c⁺CD11b⁺MHCIİH⁺) represented the major part (about 20% of leukocytes) when compared to CD8α⁺ dendritic cell (CD11c⁺CD8α⁺SiglecH⁻) and pDC (CD11c⁺ SiglecH⁺; Fig. 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 (Fig. 2C and D and Supplementary Fig. S2B). Finally, histological analyses confirmed that 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 (Fig. 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 (1520, 26).

**TApDC are functionally immature and can mediate CD4⁺ T-cell activation**

TApDC were gated based on their high expression of CD11c and Siglec-H (Fig. 2A). They expressed high levels of BST2, B220, and Ly6C while lacking Ly6G and CD11b thus confirming their identity (Fig. 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 (Fig. 3A). This phenotype resembled to the one of pDC found in naive spleen (data not shown). Furthermore, in vivo phagocytic activity was weak but similar to spleen-derived pDC (Supplementary Fig. S3) suggesting that immature TApDC may uptake tumor Ags.

---

**Figure 2.** Increased TApDC and regulatory T-cell infiltration in NEU15WT tumors escaping immunosurveillance. A and 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 and D, percentages of tumor-infiltrating pDC and Treg cells (gating strategy detailed in Supplementary Fig. S3A and S3B) were evaluated in NEU15WT and NEU15F1 tumors. **P < 0.001.** E, representative immunohistochemical staining on paraffin-embedded sections of NEU15WT tumors using anti-BST2 or anti-Foxp3 antibodies. Magnification, ×200. SiglecF⁺ m.c., myeloid cells; Neutro., neutrophil; MΦ, macrophage; Mono., monocyte.
pDC were sorted from tumor or spleen and cultured with allogeneic naive 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 (Fig. 3B and C). However, pDC maturation through TLR7 ligands, and to less extent TLR9 ligands, induced effector T-cell proliferation (Fig. 3B) as well as IFN-γ production [mean IFN-γ (pg/mL) for spleen vs. tumor pDC, respectively: Ctrl, 30 vs. 6; TLR7 ligands, 764–2312 vs. 415–2275; TLR9 ligands, 84–278 pg/mL vs. 55–88 pg/mL; Fig. 3C]. In conclusion, TApDC are phenotypically and functionally immature and may acquire abilities to activate antitumor effector T cells upon TLR7 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, IFN-α production by TApDC in response to CpG-A was strongly inhibited, with 30-fold less IFN-α than in naive spleen pDC (294.4 ± 295.9 vs. 10.65 ± 27.1; Fig. 4A). This alteration was confirmed using CpG-B/ODN-1826 with a 5-fold decrease in IFN-α production (Fig. 4A). In contrast, TApDC were as potent as naive spleen pDC to produce IFN-α in response to Flu. Other TLR7 ligands, such as CL075 or SM360320 (23), 2 synthetic TLR7 ligands, did not trigger significant IFN-α 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 (Fig. 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 (Supplementary Fig. S4). Furthermore, production of cytokine such as TNF-α 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 intraperitoneally injection from the day before tumor implantation until the experimental endpoint. A significant decrease of the tumor volume was observed upon pDC depletion from day 14 postimplantation (Fig. 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 (Fig. 5B). Specific and effective pDC depletion in the tumor upward of 80% was validated by flow cytometry (Fig. 5D) and functional IFN-α response to TLR7-L intratumoral injection at the endpoint (Fig. 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 showing 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 IFN-α inducer and a potent adjuvant (23), to revert their tumor-promoting ability. Intratumoral injections of TLR7-L (50 μg) lead to potent tumor regression when compared to vehicle-treated mice (Fig. 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 intratumoral injection, contralateral subcutaneous injection of TLR7L did not induce significant tumor regression (Fig. 6B). Both intratumoral and contralateral subcutaneous injections led however to similar range of plasmatic IFN-α levels suggesting that intratumoral route is necessary to mobilize antitumor activity of TApDC (Fig. 6C).

Analyses of intratumoral immune infiltrates exhibited an increase in leukocyte frequency as soon as 24 hours post-TLR7L injection (Supplementary Fig. S6A). Although no major changes
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 1 day before tumor implantation and every other day until tumors reached the experimental endpoint. A, data represent the mean tumor volume mean ± SEM for 20 mice. B, Kaplan-Meier survival plot of mice stratified in those with pDC depletion versus none. Data are from 1 representative out of 3 independent experiments. C and D, *in vivo* depletion of pDC was analyzed functionally (C) and phenotypically (D) by flow cytometry in tumors. C, TLR7L (10 μg) was administered by i.t. injection at experimental endpoint and serum was harvested 3 hours after for IFN-α level 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. ***, P < 0.001, **, P < 0.01.

In the frequency of most immune cells could be noticed (data not shown), a significant and specific increase in monocyte infiltration was observed (Supplementary Fig. S6B). As pDC are the most likely target of such TLR7L *in vivo*, we explored whether intratumoral TLR7L injection affected pDC frequency and function *in vivo*. Although pDC frequency remained unchanged (data not shown), TLR7L induced strong increase in MHC-II expression and costimulatory molecules at their surface (Fig. 6D). In contrast, CpG did not activate TApDC thus confirming our *in vitro* data. Specific activation of TApDC was confirmed, as neither TA-CD11b+ dendritic cell nor TA-CD11b+ dendritic cell displayed increase in MHC-II (nor CD80, CD86, data not shown) expression upon TLR7 triggering (Fig. 6D).

Finally, changes in gene expression were analyzed in NEU15WT tumors 8 hours post-intratumoral injection of TLR7L by TaqMan low-density array (TLDA) mouse immune assay (Supplementary Materials and Methods). Data are presented as fold change over the nontreated conditions and displayed as a heat map (Fig. 6E) and detailed in Supplementary Fig. S5. Genes that displayed fold changes higher than 10 were selected to highlight the most significant changes in gene expression upon TLR7 intratumoral 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*, *Cd28, Il12b, Ifnγ, Tnf, Ccl5, Cxcl10, Cxcl11*), via chemokine-mediated recruitment (*Cxc11, 2,413 ± 3,409 fold changes; Cer7, 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 IFN-γ–induced genes (*Cxcl10, Cdl5, 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 coinjected with TLR7L (Supplementary Fig. S5A). In parallel, an increase in type I IFN genes (*Ifna* and *Mx1*) was seen in samples treated by TLR7L by quantitative PCR (Supplementary Fig. S5B).

Altogether these data showed 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**

To explore whether TLR7L antitumoral activity was mediated by pDC, pDC were depleted the 2 days before TLR7L injection. As previously observed with a dose of 50 μg, 10 μg of TLR7L also lead to a significant decrease of tumor volume (day 35; Fig. 7A). pDC depletion in such short-term schedule led to a delay in tumor growth as previously observed (Fig. 7A). As previously observed with a dose of 50 μg, 10 μg of TLR7L also lead to a significant decrease of tumor volume (day 35; Fig. 7A). pDC depletion in such short-term schedule led to a delay in tumor growth as previously observed (Fig. 7A).
the antitumor effect of TLR7L in vivo (Fig. 7C). The efficiency of pDC depletion was confirmed phenotypically (data not shown) and functionally at the type I IFN systemic plasmatic level (Fig. 7B).

We then assessed whether inhibition of type I IFN signaling affected TLR7L activity. For that matter, anti-IFNAR1 or control antibodies were coadministered intratumoral with TLR7L (10 μg). IFNAR inhibition resulted in a significant decrease of TLR7L antitumoral activity showing the requirement for type I IFN in TLR7L activity (752.1 ± 135.3 mm³ for TLR7L vs. 1,392.1 ± 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. 1,826.6 ± 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 IFN-α production in contrast with TLR7L (data not shown). Surprisingly, pDC depletion altered Cpg antitumoral activity (Fig. 7D), however inhibition of type I IFN signaling did not (Fig. 7E). These results suggest that pDC indirectly affect Cpg activity through a distinct type I IFN-independent mechanism.

Altogether, those data show 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 show 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, 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). Although pDC hyporesponsiveness to TLR9 ligands might be explained by receptor downregulation (17, 28), we showed 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 to avoid immune alert by putative endogenous ligands for TLR9 within the tumor microenvironment, such as described in autoimmune disorders (30–32).

Second, we showed 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. Although 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 (Fig. 7B and D). The use of a pDC-deficient mouse model (33) will formerly show 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 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 IFN-α-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 intratumoral 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, showing the central role for TApDC in TLR7L-mediated antitumor response. Importantly, we showed that this therapeutic activity is mediated by locally induced and not systemic type I IFN. This points toward 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 CD80/CD86 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.

Although we showed both in vitro and in vivo that CpG could not activate TApDC, this TLR9L unexpectedly induced tumor regression. Although 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 (Supplementary Fig. S6b) that was reduced upon pDC depletion (data not shown). Although 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 in 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: I. Le Mercier, J. Vlach, J.-Y. Blay, N. Bendriss-Vermare, I. Puisieux, N. Goutagny


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Le Mercier, D. Poujol, A. Sanlaville, M. Gobert, I. Treilleux, B. Dubois, J.-Y. Blay, I. Puisieux, N. Goutagny

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Le Mercier, D. Poujol, J.-Y. Blay, N. Bendriss-Vermare, C. Caux, I. Puisieux, N. Goutagny

Writing, review, and/or revision of the manuscript: I. Le Mercier, J.-Y. Blay, N. Bendriss-Vermare, C. Caux, I. Puisieux, N. Goutagny

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Poujol, A. Sanlaville, V. Sirisak, J. Vlach, I. Puisieux, N. Goutagny

Study supervision: J.-Y. Blay, C. Caux, I. Puisieux, N. Goutagny

Acknowledgments

The authors thank S. Goddard-Léon and M. Pratviel for technical support, M. Faturu and A. Besse for previous advice on statistical analysis, and Janssen Infectious Diseases-Diagnostics BVBA for providing SM360320.

Grant Support

This work is supported by The BCRF, Ligue Contre le Cancer, Institut National du Cancer (INCa AC1-63-04, METESCAPE, ANR-10-LABX-0061, ARC 3364, EML 2009, LYRIC, INCa_466), and Lyon Biopole.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 7, 2012; revised April 25, 2013; accepted May 11, 2013; published OnlineFirst May 30, 2013.
References


Tumor Promotion by Intratumoral Plasmacytoid Dendritic Cells Is Reversed by TLR7 Ligand Treatment

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

Cancer Res  Published OnlineFirst May 30, 2013.