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Breast cancer-derived transforming growth factor-β and tumor necrosis factor-α compromise interferon-α production by tumor-associated plasmacytoid dendritic cells

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We previously reported that plasmacytoid dendritic cells (pDCs) infiltrating breast tumors are impaired for their interferon-α (IFN-α) production, resulting in local regulatory T cells amplification. We designed our study to decipher molecular mechanisms of such functional defect of tumor-associated pDC (TApDC) in breast cancer. We demonstrate that besides IFN-α, the production by Toll-like receptor (TLR)-activated healthy pDC of IFN-β and TNF-α but not IP-10/CXCL10 nor MIP1-α/CCL3 is impaired by the breast tumor environment. Importantly, we identified TGF-β and TNF-α as major soluble factors involved in TApDC functional alteration. Indeed, recombinant TGF-β1 and TNF-α synergistically blocked IFN-α production of TLR-activated pDC, and neutralization of TGF-β and TNF-α in tumor-derived supernatants restored pDCs' IFN-α production. The involvment of tumor-derived TGF-β was further confirmed *in situ* by the detection of phosphorylated Smad2 in the nuclei of TApDC in breast tumor tissues. Mechanisms of type I IFN inhibition did not involve TLR downregulation but the inhibition of IRF-7 expression and nuclear translocation in pDC after their exposure to tumor-derived supernatants or recombinant TGF-β1 and TNF-α. Our findings indicate that targeting TApDC to restore their IFN-α production might be an achievable strategy to induce antitumor immunity in breast cancer by combining TLR7/9-based immunotherapy with TGF-β and TNF-α antagonists.

Type I interferons (IFNs α, β, ω) are central players in the protection against viral infections and cancer development through their direct effects on infected and tumor cells and their ability to regulate antiviral and antitumor immune responses. Indeed, they prime innate and adaptive immune cells and promote the crosspresentation of viral and tumor antigens to $CD8^+$ T cells.[1–3] Recent studies have also clearly demonstrated a crucial role for type I IFNs in tumor immunosurveillance and immune rejection through the activation of conventional $CD8\alpha^+$ dendritic cell (DC).[1,2] Thus, there is a clear advantage for infected and tumor cells to suppress type I IFN production to escape immune responses.

Plasmacytoid dendritic cells (pDC) are the main type I IFN producers in humans and mice.[4] They play a key role in

Key words: plasmacytoid dendritic cells, breast cancer, type I IFNs, TGF-β, TLR ligands

Abbreviations: BDCA: blood dendritic cell antigen; BT: breast tumor; ODN: oligodeoxynucleotide; pDC: plasmacytoid dendritic cells; TApDC: tumor-associated pDC; TLR: Toll-like receptor; TumSN: tumor supernatant

Additional Supporting Information may be found in the online version of this article.

*C.C and N.B.-V. contributed equally to this work

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What's new?

Human plasmacytoid dendritic (pDC) cells are known to infiltrate tumors but are functionally impaired, and their infiltration is associated with poor prognosis for tumor patients. The present study uncovers cooperation between TGF-beta and TNF-alpha as a major in vivo mechanism blocking type I interferon production by tumor-associated pDCs through inhibition of IRF7 signaling. The authors propose that a combination of TGF-beta/TGF-beta receptor antagonists with TLR7/9 agonists may restore tumor-associated pDCs' innate immune functions thus restoring a more effective anti-tumor immunity and leading to innovative new treatments for tumors such as breast cancer.

antiviral immune responses through their massive production of IFN-α in response to both viral DNA and RNA recognized by Toll-like receptors (TLRs) 9 and 7, respectively.[4] Besides their role in antiviral immunity, pDCs are also involved in the pathogenesis of autoimmune disorders. Indeed, complexes of self-nucleic acids with autoantibodies and/or antimicrobial peptides trigger a sustained pDC production of type I IFN exacerbating the disease.[5] The phosphorylation and nuclear translocation of the transcription factor IFN regulatory factor (IRF)-7 after TLR engagement is critical to initiate the transcription of type I IFNs in pDC.[6]

We and others have previously demonstrated that human pDCs infiltrate tumors but are functionally impaired[7–11] and associated with poor prognosis.[8,10,12] Moreover, we recently reported that (*i*) the deleterious impact of breast tumor-associated (TA) pDC on clinical outcome[10] is owing to their defect in IFN-α production that contributes to regulatory T cells amplification[11] and (*ii*) tumor-derived soluble factors are responsible for breast tumor-associated pDC (TApDCs)' dysfunction,[11] as in ovarian[8] and head and neck[7] cancers. However, the inhibitory mediators as well as the molecular mechanisms involved in this TApDC functional inhibition in breast cancer remain unknown.

Herein, we show that tumor-derived transforming growth factor-β (TGF-β) synergizes with tumor necrosis factor-α (TNF-α) to suppress specifically type I IFNs (α/β) and TNF-α production by TLR-activated pDC through the inhibition of IRF-7 expression and nuclear translocation. Thus, our results suggest that neutralization of TGF-β and TNF-α in association with TLR7/9 agonists might be a potential therapeutic approach to restore TApDCs' functions in primary breast cancer.

Material and Methods

Patients, human tissue samples and blood

Fresh tumors from breast cancer patients, discarded human tonsils and human healthy blood were obtained as described previously.[11] For more details, see Supporting Information **Materials and Methods**.

Isolation and stimulation of pDC and mDC

Breast tumors (BTs), tonsils and blood were processed as described previously.[11] pDC and mDC were purified as described in Supporting Information **Materials and Methods**. Healthy pDCs were cultured at 2–5 × 10^4/well in 100 μL in different conditions for overnight and then stimulated with TLR ligands for 24 hr before cytokines and chemokines were measured in the supernatants by enzyme-linked immunosorbent assay (ELISA). Blocking experiments were performed using neutralizing antibodies (Abs) against TGF-βs, TNF-α and IL-10. For more details, see Supporting Information **Materials and Methods**.

Tumor tissue immunofluorescence

Frozen BT sections were fixed in 4% paraformaldehye (PFA) and stained with monoclonal antibodies (mAbs) against BDCA2 (mouse IgG1, Dendritics, Lyon, France) (10 μg/mL) and phospho-Smad2 (rabbit IgG, Cell Signaling Technology, Boston, Massachusets) (1 μg/mL) revealed with Alexa-488-conjugated goat anti-mouse IgG1 (1:400) and Alexa 555-conjugated goat anti-rabbit IgG (1:400) Abs (Molecular Probes) and nuclei coloration with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Images from ProLong® gold anti-fade reagent (Molecular Probes, Eugene, Oregon) mounted slides were acquired using confocal system (Zeiss Axioplan2 LSM510 Confocal microscope) at a magnification of 62× (Axiovision 4.0v4.6.1.0 program). The percentage of cells coexpressing BDCA2 and phospho-Smad2 was evaluated by calculating the "$BDCA2^+$ phospho-$Smad2^+$ cells to total $BDCA2^+$ cells" ratio after cell counting on three different areas of three different tumor sections.

IRF-7 staining

In brief, 2 × 10^4 pDCs were seeded on poly-L-lysine (Sigma-Aldrich, Steinhein, Germany) (1 μg/mL) precoated teflon-printed slides (Immuno-Cell International, Mechelen, Belgium) and stimulated for 3 hr with 100 HAU/mL inactivated Flu virus, fixed in 4% PFA, permeabilized in −20°C methanol, and dried at room temperature for 1 hr. After blocking, rehydrated slides were stained with anti-IRF-7 mAb (rabbit IgG, Santa Cruz Biotechnology, Dallas, Texas) used at 20 μg/mL for 1 hr followed by Alexa-488-conjugated goat antirabbit Ig (Molecular Probes) (1:500). Nuclei were colored with DAPI. Slides were mounted and images were acquired as mentioned previously.

Western blot analysis

The expression of phospho-Smad2, total Smad2/3, and β-actin proteins was detected using rabbit polyclonal anti-

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phospho-Smad2, rabbit monoclonal anti-Smad2 (Cell Signalling Technology) and mouse monoclonal β-actin (Sigma) Ab.

Real-time quantitative PCR analysis

Total RNA was isolated using RNeasy Kit (Qiagen, Hilden, Germany). cDNA was synthesized with the SuperScript III (Invitrogen, Eugene, Oregon) reverse transcriptase synthesis kit. Quantitative real-time PCR was performed on the 7000 Real-Time PCR System (Applied Biosystems, Branchburg, New Jersey) using the Mesagreen qPCR Master Mix Plus for SYBR® Assay (Eurogentec, San Diego, California). Relative quantification was performed using standard curve analysis. All gene expression data were normalized with β2-microglobulin/cyclophilin B geometric mean. The primers used and their sequences are listed in Supporting Information Table S1.

Statistical analyses

Statistical significance of differences was determined by the non parametric unpaired Mann–Whitney *U*-test and the parametric Student's *t*-test for paired or unpaired samples when appropriate. Correlations were evaluated using the non-parametric Spearman test. Differences were considered significant for *p*-values of <0.05 and are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Results and Discussion

BT environment specifically blocks type I IFNs and TNF-α production by healthy donor TLR-activated pDC

We recently reported that breast TApDCs are impaired for their IFN-α production.[11] To decipher mechanisms mediating such dysfunction, we first developed an *in vitro* culture model that mimics the *in vivo* BT microenvironment by exposing healthy pDC to supernatants derived from single-cell suspensions of primary BT (TumSN) prior stimulation with TLR ligands. The majority (>75%) of TumSN strongly (inhibition, >40%) inhibited pDCs' IFN-α production in response to inactivated Flu virus (TLR7-L) (Fig. 1*a* and Supporting Information Fig. S1) (mean inhibition: 53.3 ± 23.6 and 50.2 ± 18.6%, respectively) and CpG-A (TLR9-L) (Supporting Information Fig. S1). The ability of breast TumSN to alter pDC-derived IFN-α secretion in response to both TLR ligands was strongly correlated ($r^2 = 0.832$, $p < 0.01$) (Fig. 1*b*). Importantly, although the production of IFN-β and TNF-α was also inhibited (mean inhibition: 57.2 ± 23.6 and 55.4 ± 16.5%, respectively), IP-10/CXCL10 and MIP-1α/CCL3 levels remained unaffected (Fig. 1*a* and Supporting Information Fig. S1). Furthermore, intensity of IFN-α inhibition by TumSN correlates with TNF-α and IFN-β inhibition (Supporting Information Fig. S2), suggesting that common soluble mediators produced in inhibitory TumSN block both TLR7 and TLR9 pathways and a common set of selected cytokines. Similarly, healthy pDCs primed in head and neck[7,13] or ovarian[8] TumSN were shown to be strongly inhibited for IFN-α production mainly after TLR9 stimulation.

TGF-β and TNF-α released by BT mediate the inhibition of IFN-α production by pDC

To address the mechanisms of IFN-α inhibition in TApDC, we initially examined the role of commonly known tumor-associated immune modulators. First, breast TumSN contained significant levels of total TGF-β1 (820–2,740 pg/mL), active TGF-β1 (25.8–752 pg/mL), active TGF-β2 (22.2–124.1 pg/mL), TNF-α (0–2,276 pg/ml) and IL-10 (0–1,004 pg/mL) (Fig. 1*c*). Second, we observed that 16-hr pretreatment with TGF-β1, TNF-α, or IL-10, used at IC50 that was calculated from dose–response data on IFN-α inhibition (dose–response experiment, Supporting Information Fig. S3A), significantly inhibited the secretion of IFN-α, IFN-β and TNF-α ($p < 0.05$) (Fig. 2*a* and Supporting Information Fig. S3A) by Flu virus-activated pDC. In addition, IL-10 but neither TGF-β nor TNF-α strongly inhibited IP-10/CXCL10 and MIP-1α/CCL3 in a significant manner ($p < 0.05$) (Fig. 2*a* and Supporting Information Fig. S3A), excluding IL-10 contribution in the functional alteration of pDC by TumSN. GM-CSF, M-CSF, IL-6 and VEGF also detected in breast TumSN (data not shown) and reported to modulate myeloid (m)DC in tumor environment,[14,15] had no impact on IFN-α production by TLR-activated pDC (data not shown). Third, using validated neutralizing reagents (data not shown), TumSN-mediated suppression of pDC-derived IFN-α secretion was totally reversed by blocking both TGF-β and TNF-α, whereas blocking TNF-α or TGF-βs independently but not IL-10 showed only a partial effect (Fig. 2*b*). This is consistent with the synergistic effect of TGF-β1 and TNF-α in inhibiting IFN-α production by Flu virus-activated pDC (Fig. 2*a* and Supporting Information Figs. S3B and C). Importantly, TumSN containing high levels of both TGF-β1 and TNF-α inhibited more strongly pDC-derived IFN-α secretion than TumSN with high levels of only one cytokine ($p = 0.002$) or low levels of both cytokines (Fig. 1*d*) ($p < 0.001$). Thus, BT-derived TGF-β and TNF-α cooperate to suppress IFN-α production by TApDC. This is in accordance with the previous observations, showing that (*i*) TNF-α negatively regulates the production of IFN-α by TLR-activated pDC,[16] (*ii*) TGF-β conditions pDC for low type I IFN production in peyer patches[17] and spleen,[18] (*iii*) intratumoral levels of IFN-α and TGF-β1 are inversely correlated in breast cancer patients[19] and (*iv*) TGF-β suppresses IFN-α production by pDC in head and neck[13] and ovarian cancers,[8] in association with PGE-2 and TNF-α, respectively.

The biological effects of TGF-β are mediated through the TGF-β receptors I and II that activate the Smad signaling cascade, leading to Smad2 and Smad3 phosphorylation.[20] Consistent with TGF-β receptors expression (Supporting Information Fig. S4), we observed by Western blot that Smad2 phosphorylation in healthy pDC exposed to TGF-β *in vitro* (Fig. 2*c*). Importantly, phosphorylated Smad2 was detected in the nuclei of $BDCA2^+$ TApDC (71.4% of $BDCA2^+$ cells are $phosphoSmad2^+$, $n = 3$ independent tumors) by immunofluorescence (IF) on BT sections (Fig. 2*d*). These observations demonstrate that TApDCs are exposed to TGF-βs released in BT environment and suggest a role for Smad signaling in

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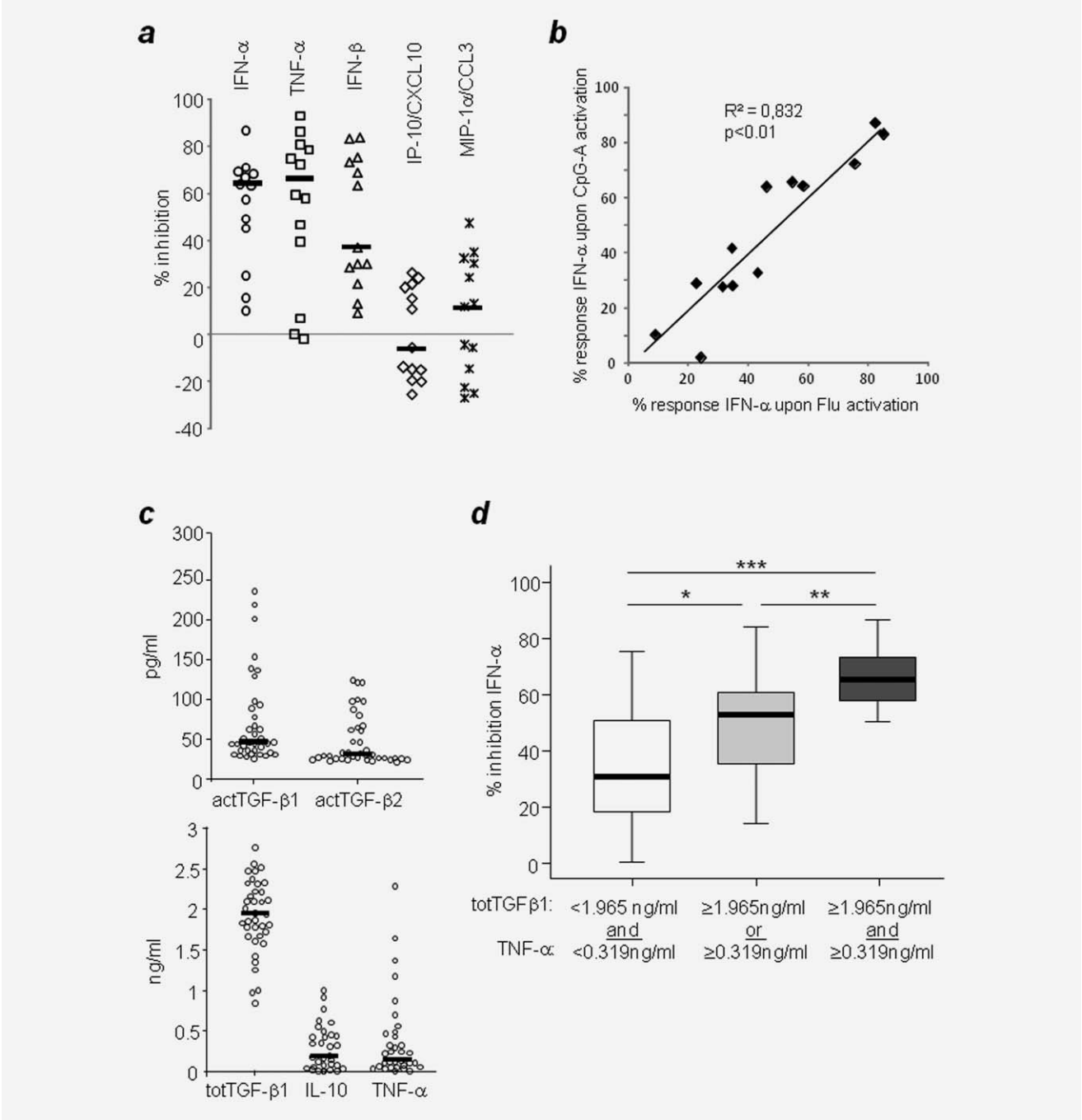

Figure 1. Inhibition of TLR-stimulated pDC-derived IFN-α secretion in the presence of breast TumSN correlates to TGF-β and TNF-α levels. (*a*) Healthy pDCs were pretreated with IL-3 ± TumSN (25%) derived from 13 patients for overnight and then stimulated with Flu virus for 24 hr. The production of IFN-α, TNF-α, IFN-β, IP-10/CXCL10 and MIP-1α/CCL3 was quantified by ELISA. Data are expressed as the percentages of inhibition, by subtracting the quantity of each cytokine detected in TumMSN alone in the absence of pDC. Data are representative of more than four independent experiments, representing more than 30 independent TumSN. Horizontal bars represent the median. (*b*) Correlation between the percentage of IFN-α response after pDC-activation by Flu virus and CpG-A in the presence of TumSN. Each dot represents an independent TumSN, $n = 13$. Data are expressed as the percentages of IFN-α response in the presence of TumSN compared to that response in control conditions. (*c*) Levels of active TGF-β1 and TGF-β2, total TGF-β1, IL-10 and TNF-α in TumSN (ELISA). Each symbol represents an independent TumSN and horizontal bars represent the median. (*d*) Box plot sets of the percentages of IFN-α inhibition in the presence of TumSN compared to control conditions in three groups of TumSN characterized by their amounts of total TGF-β1 and/or TNF-α. The median for total TGF-β1 (1.96 ng/mL) and TNF-α (0.32 ng/mL) levels in the whole group of TumSN was used as cutoff to define the three groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; nonparametric Mann–Whitney *U*-test.

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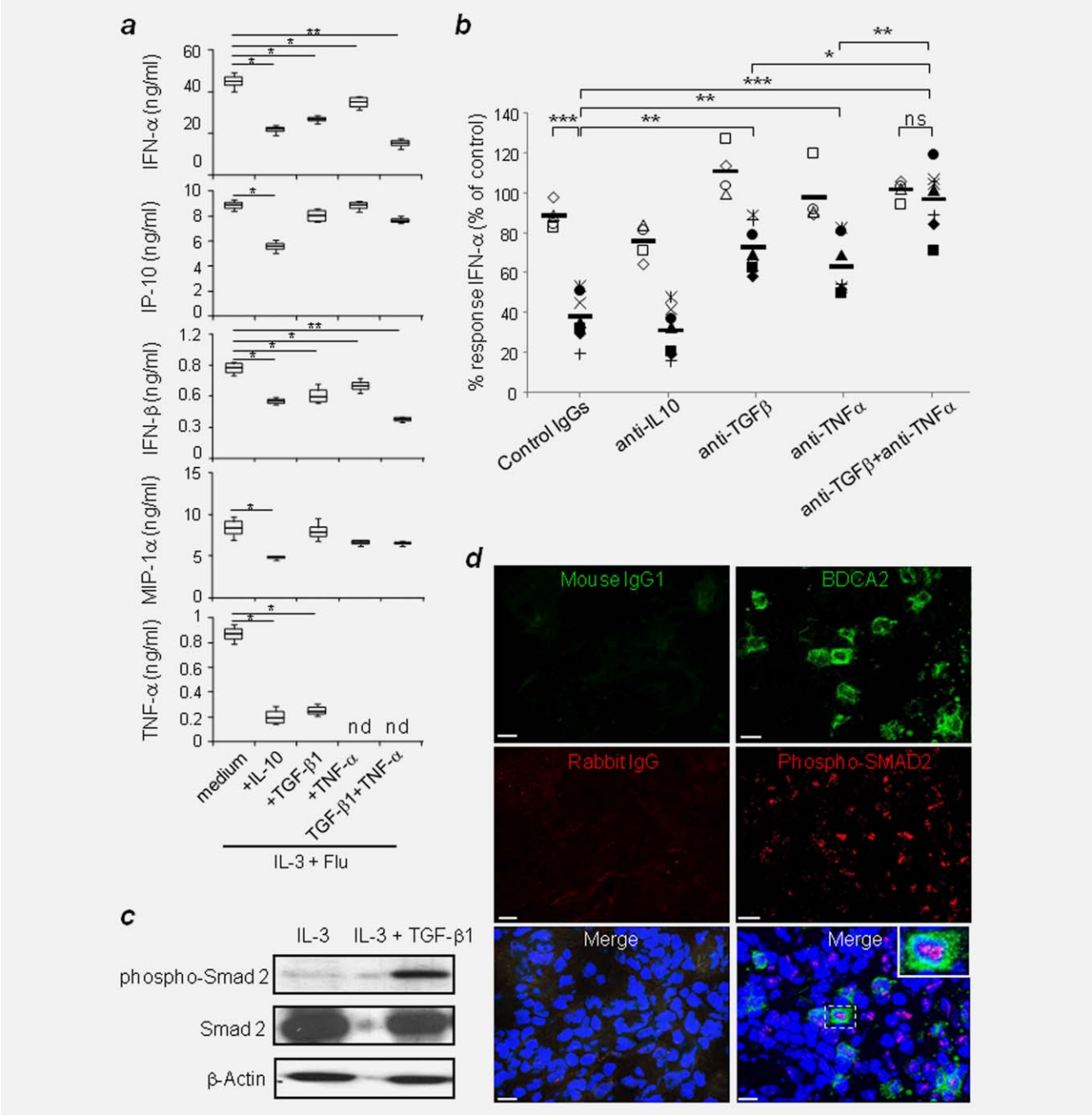

Figure 2. BT environment mediates inhibition of IFN-α production by TLR-activated pDC *in vitro* and TApDC *in vivo* through TGF-β1 and TNF-α. (*a*) Healthy pDCs were pretreated with IL-3 ± TGF-β1 (0.01 ng/mL), TNF-α (0.5 ng/mL) and IL-10 (0.25 ng/mL) for 16 hr prior Flu virus activation for 24 hr. Data are presented as box plot sets of cytokine production for each culture condition. $*p < 0.05$, $**p < 0.01$; nonparametric Mann–Whitney *U*-test for unpaired samples. (*b*) Healthy pDCs were cultured as described in Figure 1 in the presence (black symbols) or not (white symbols) of TumSN (each symbol represents an independent TumSN/patient, $n = 7$) and in the presence or absence of anti-IL-10, anti-TNF-α, anti-TGF-β blocking Abs (10 μg/mL). IFN-α production was measured by ELISA. IFN-α responses were normalized to 100% for pDC culture in the absence of any TumSN and any mAbs. Horizontal bars represent the mean. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; parametric Student's *t*-test for paired (when each Ab treatment was compared to the control IgGs in the presence of TumSN) or unpaired (when TumSN were compared to medium condition) samples. (*c*) Immunoblot detection of Smad2 and its phosphorylated form in healthy pDC cultured for 3 hr with IL-3 ± TGF-β (1 ng/mL). Results are representative of three independent experiments. (*d*) Tumor sections were stained for BDCA2 (green) and phospho-Smad 2 (red). Nuclei were identified using DAPI (blue). Data are representative of three independent BT. Scale bars represent 10 μm.

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TGF-β-mediated TApDC inhibition, as already demonstrated for Smad3 in mice.[18]

BT environment as well as TGF-β1 + TNF-α impair pDCs' IFN-α production by blocking IRF-7 expression and nuclear translocation

We next studied how TGF-β and TNF-α are interfering with IFN-α signaling pathways in TLR-activated pDC. We did not observe a general downregulation of TLR7 and TLR9 expression in TApDC by flow cytometry (Fig. 3*a*) or in healthy pDC exposed to TumSN by quantitative PCR (Fig. 3*b*). On the other hand, we observed a downregulation of constitutive IRF-7 mRNA expression in healthy pDC exposed to TGF-β1 + TNF-α and inhibitory TumSN (TumSN #7, #9 and #10) (Fig. 3*c*), but not to noninhibitory TumSN #3, consistently with their effect on IFN-α secretion (Fig. 2*b*).

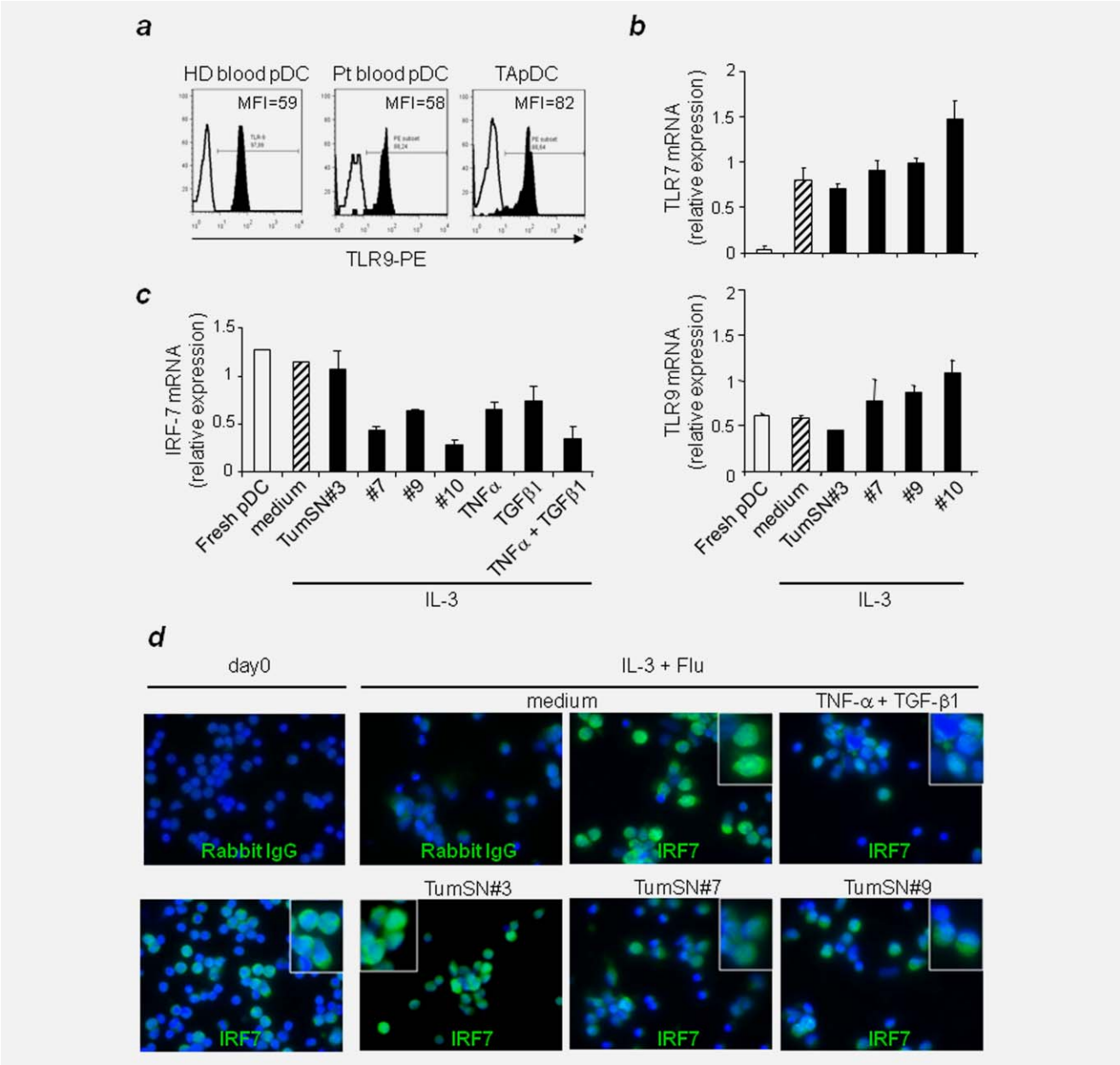

Figure 3. BT environment and TNF-α + TGF-β1 block expression, upregulation and nuclear translocation of IRF-7 in pDC. (*a*) Flow cytometry analysis of TLR9 intracellular expression in healthy donor (HD) or patients (Pt)' blood pDC and TApDC. Representative histograms of three separate experiments performed on three different donors are shown. (*b* and *c*) Relative TLR7, TLR9 and IRF-7 mRNA expression in freshly isolated healthy pDC and pDC cultured for 16 hr with IL-3 either alone (medium) or in the presence of noninhibitory TumSN#3 or inhibitory TumSN#7, #9, #10 or TNF-α ± TGF-β1 (1 ng/mL). Data are shown as a mean of relative expression ± SD from duplicate values of three independent donors. (*d*) Healthy blood pDC pretreated or not with TNF-α + TGF-β1 (1 ng/mL) or three different TumSN (25%) for 16 hr prior Flu virus activation (3 hr) were stained for IRF-7 (green). Nuclei were identified using DAPI (blue). Data are representative of three independent experiments.

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Furthermore, IF stainings showed that IRF-7 protein, localized in the cytoplasm of resting pDC, was upregulated and translocated in the nucleus after 3-hr stimulation with Flu virus (Fig. 3*d*). This process was markedly decreased in the presence of inhibitory TumSN #7 and #9 or TGF-β1 + TNF-α (Fig. 3*d*) but not with noninhibitory TumSN#3. Therefore, BT environment through TGF-β1 + TNF-α affects both expression and activation of the transcription factor IRF-7, leading to reduced ability of TLR-stimulated TApDC to produce IFN-α. This may result from interference between signaling pathways downstream TLR and TGF-βR as we did not find any Smad-binding elements in the promoter of *Irf7* gene. Our observations are in contrast with a previous report in head and neck cancer, suggesting that tumor-induced downregulation of TLR9 expression may contribute to impaired IFN-α production by CpG-activated TApDC.[7] Moreover, this specific interference with the IRF-7 signaling pathway controlling IFN-α production in pDC is consistent with the lack of inhibition by breast TumSN on TLR-triggered pDCs' maturation and production of IP-10/CXCL10, MIP-1α/CCL3 and IL-8, showing the absence of NFκB signaling dysregulation.[21] Nevertheless, it remains to be explored why TNF-α production, supposed to be NFκB-dependent, is concomitantly inhibited in pDC exposed to the BT environment.

Tumor escape from immune recognition represents one of the key issues to be faced in cancer therapy. We previously proposed that BT inhibits type I IFN secretion by TApDC to escape their physiological role in immunosurveillance and favor a tolerogenic environment, and thus preventing an effective antitumor response and facilitating tumor progression.[11]

Indeed, pDCs that are chronically activated by (LL37/self-nucleic acids) complexes produced excessive levels of IFN-α that participated to the development/maintenance of noninfectious autoimmune/inflammatory pathologies.[22,23] In contrast, in BT we observed that TApDCs are strongly inhibited for their IFN-α production upon stimulation with exogenous TLR-L *in vitro*. As (*i*) tumors have been shown to express LL37[24] and (*ii*) endogenous danger signals such as self-nucleic acids[25] are released from dying tumor cells, it is tempting to speculate that, upon (LL37/self-nucleic acids) complexes recognition TApDC might contribute to tumor immunosurveillance through type I IFNs production. This is consistent with the partially activated phenotype of breast TApDC. Thus, the tumor has evolved mechanisms to inhibit type I IFN secretion by TApDC to prevent an effective antitumor response and favor tumor progression. This hypothesis is currently under investigation but it is supported by recent studies, showing that (*i*) type I IFN is selectively required by DC for immune rejection of tumors[1,2] and (*ii*) the type I IFN signature is predictive of responses to anthracyclines in breast cancer patients.[26]

Therefore, the dissection of the molecular mechanisms controlling the innate TApDC functions should lead to new ways to manipulate these cells for cancer therapy. Our study now identifies TGF-β and TNF-α as novel targets for restoring type I IFN production by TApDC. As (*i*) TLR9 (CpG ODN) and TLR7 ligands that specifically target human pDC are currently evaluated in clinical trials for immune stimulation in tumor patients,[27] (*ii*) drugs that target TGF-β or its receptors' signaling are currently in development[28] and (*iii*) pDCs are mandatory for the antitumor response mediated by imiquimod (TLR7-L) in a murine model of melanoma through IFN-α production and acquisition of cytotoxic properties,[29] our results suggest that restoring TApDC-derived type I IFN using TGF-β antagonists (±TNF-α inhibitors) combined to TLR7/9 activation represent a promising new therapeutic strategy in localized BT.

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Author contributions

V.S., J.-Y.B., C.C. and N.B.-V designed research; V.S., N.V., N.G., M.M., S.R. and S.T. performed research; I.T., T.B. and S.I.L.-G. provided patients' samples; V.S., N.V., N.G., C.M.-C., J.-Y.B., C.C. and N.B.-V analyzed data; S.I.L.-G., C. D.-D. and J.-Y.B. provided helpful discussions; V.S., C.C. and N.B.-V. wrote the article.

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Short Report