Brief Correspondence

Conventional and PD-L1-expressing Regulatory T Cells are Enriched During BCG Therapy and may Limit its Efficacy

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Intravesical instillation of Bacillus Calmette-Guérin (BCG) is used since several decades as a standard treatment for non-muscle invasive bladder cancer (NMIBC). Although it remains as one of the most effective treatments, 20–30% of patients experience early recurrence despite BCG therapy and they eventually undergo cystectomy [1]. The role of the immune system in the BCG therapeutic effect is undoubtedly crucial as several predictive/prognostic markers belong to various immunologic components [2]. Notably, immune parameters that may impact clinical response to BCG include cytokines released locally [2,3], the existence of preexisting immunity to BCG [4], the Th1 versus Th2 milieu...
[5], as well as the local balance between T lymphocytes and myeloid suppressor cells [6]. In the latter study, we found that patients showing locally a higher number of T cells than of myeloid-derived suppressor cells responded better to BCG therapy. However, the immune response is multifaceted so that T cells may themselves include subpopulations of immunosuppressive cells. We, thus, investigated whether and to what extent urine-infiltrating T cells could encompass: (1) conventional T regulatory cells (cTregs), and/or (2) PD-L1+ Tregs, a novel regulatory subset [7]. cTregs were previously assessed in NMIBC by CD25 or FoxP3 immunohistochemical staining and found at elevated levels in patients with BCG failure [8,9]. However, PD-L1+ Tregs, recently described as key players in the therapeutic effect of interferon (IFN)-β treatment for multiple sclerosis autoimmune disease [7], have not been investigated to our knowledge in the context of cancer.

In this prospective study, we investigated the putative involvement of conventional and PD-L1+ Tregs in 17 patients receiving BCG therapy. We measured cTregs (CD3+CD4+CD25+CD127low) by flow cytometry (Fig. 1A and Supplementary Fig. 1) in peripheral blood mononuclear cells (PBMCs) from healthy donors (HD) and urothelial cancer (UCa) patients, as well as in the urine of patients during BCG therapy (pre- and post-BCG samples) by flow cytometry (Fig. 1B). Notably, cTregs were substantially enriched among urine CD4 T cells during BCG treatment, with no difference in pre- versus post-BCG instillation samples (Fig. 1C). Besides, circulating cTregs levels were stable from before to 4–6 wk after starting intravesical treatments (Supplementary Fig. 2). Thus, data argue for a BCG-independent local enrichment in cTregs. In vitro coculture of PBMC from HD with the T24 bladder cancer cell line increased the cTregs proportion among CD4 T cells (Figs. 1D and 1E), in contrast to BCG (data not shown). Accordingly, Loskog et al [10] found cTregs infiltration in invasive and superficial bladder tumor tissues, apart from BCG therapy, and showed that tumor cells produced transforming growth factor-β that could induce cTregs.

We next investigated the presence of the new regulatory PD-L1+ CD4 T-cell subset (Fig. 2A). While PD-L1+ Tregs were not clearly detectable in the blood of HD and UCa patients, they were found at high, albeit variable, levels in the urine of patients during BCG treatment (Fig. 2B). In contrast to cTregs, we observed a higher frequency of PD-L1+ Tregs in post-BCG instillation specimens than in pre-BCG urine samples (Fig. 2C) or in pre-therapy samples, only available for five patients (Fig. 2D). Of note, PD-L1 Tregs from urine samples expressed the transcription factor FoxA1 (Forkhead Box A1), thus confirming their regulatory phenotype (Supplementary Fig. 3), as previously shown [7]. Moreover, in vitro co-culture experiments showed that (1) T24 bladder tumor cells alone induced only low levels of PD-L1+ Tregs, (2) BCG alone had a limited yet significant ability to induce PD-L1+ Tregs, and (3) combined T24 and BCG showed a strong synergistic
Fig. 2 – PD-L1+ Tregs are specifically induced locally during BCG therapy and levels of regulatory T cells associate with tumor recurrence. PBMCs and urine cells were analyzed by flow cytometry. (A) Cells were gated on live CD3+ leukocytes and PD-L1+ Tregs were defined as CD4+PD-L1+; (B) PD-L1+ Tregs frequency among CD4 T cells in PBMCs from HD (n = 9), patients with bladder cancer (UCa, n = 11 NMIBC before or during BCG treatment and n = 7 MIBC), and in the urine samples from patients receiving BCG therapy (21 pre- and 31 post-BCG samples); (C) PD-L1+ Tregs levels in samples before (pre) versus 4 h after (post) BCG instillations; (D) For five patients, enough cells were present in the urine before starting the course of BCG therapy to measure PD-L1+ Tregs, the levels of which were compared to the mean levels measured in post-BCG samples from the corresponding patients; (E and F) PBMCs from HD were co-cultured for 4 d with T24 bladder cancer cells, with BCG, with both T24 cells and BCG, or with medium alone as a control. (G) Supernatants of PBMCs and bladder cell lines were analyzed for IFN-β levels. (H) Supernatants from BCG-stimulated cell lines were analyzed for the presence of PD-L1+ Tregs. (I) Supernatants from BCG-stimulated cell lines were analyzed for the presence of PD-L1+ Tregs. (J) Urine immunosuppressive (IS) score (based on cTregs and PDL1+ Tregs).
effect leading to high levels of PD-L1+ Tregs (Figs. 2E and 2F). Similar results were obtained when using UROtsa and Bu68.8 cell lines (Supplementary Figs. 4A and 4B). Next, we tested the effect of PD-1/PD-L1 blockade in an autologous T-cell proliferation assay in the presence of PD-L1+ CD4 T cells. To this end, BCG/T24-stimulated or unstimulated CD4 T cells (ie, containing or not PD-L1-expressing cells, respectively, as shown in Fig. 2F) were magnetically sorted and co-cultured for 4 d, with autologous CFSE-labeled total T cells in the presence of antibodies blocking the PD-1/PD-L1 interaction. We observed that while blocking PD-1/PD-L1 interaction had no effect on T-cell proliferation, when co-cultured with untreated CD4 T cells, the blocking significantly increased CD4 and CD8 T-cell proliferation when co-cultured in the presence of BCG/T24-stimulated CD4 T cells (Supplementary Fig. 5). Together with previous reports [11,12], these results suggest that blocking PD-1/PD-L1 interaction may increase immune-related BCG activity.

IFN-β was recently described as a key inducer of PD-L1+ Tregs [7]. Indeed, while BCG-stimulated PBMCs produced low levels of IFN-β, bladder cell lines (derived from healthy or tumor urothelium) produced substantial amounts of IFN-β upon BCG stimulation (Fig. 2G). In addition, supernatants from all BCG-stimulated bladder cell lines were able to induce PD-L1+ Tregs (Fig. 2H), and interfering with the IFN-β signaling using a blocking antibody reduced this effect (Fig. 2I). Thus, these data suggest that BCG infection of urothelial cells, whether malignant or not, leads to the induction of PD-L1+ Tregs, partially via an IFN-β-dependent mechanism. Notably, large-scale analysis from the Cancer Genome Atlas database of patients with muscle-invasive bladder cancer (MIBC) showed that tumor samples with detectable IFN-β mRNA (142/404 samples) had higher PD-L1 mRNA levels (Supplementary Fig. 6A), with a weak but significant positive correlation between IFN-β and PD-L1 transcripts levels (Supplementary Fig. 6B).

As conventional (CD25+CD127low) and PD-L1+ Tregs were found to represent a sizeable compartment of urine-infiltrating T lymphocytes during BCG therapy, we next investigated whether levels of these immunoregulatory cells may differ between patients showing rapid tumor recurrence following BCG therapy. We, thus, prospectively followed the recurrence-free survival (RFS) time following BCG treatment (median follow-up was 12 mo). Taken independently, neither cTregs nor PD-L1+ Tregs levels showed significant prognostic value (data not shown). Therefore, we calculated a “urine immunosuppressive (IS) score” taking levels of both subsets into account (IS score calculation is described in Supplementary Material and Methods). Interestingly, while the IS score was similar in patients showing pTa or pT1 tumors, patients with a high IS score showed significantly shorter RFS (median RFS was 3.5 mo, while it was not reached in patients with low IS scores; Fig. 2J).

Immune checkpoint blockade strategies that target regulatory pathways to restore and enhance T-cell antitumor activity are revolutionizing cancer therapy [13]. Notably, the use of antibodies blocking the PD-1/PD-1 axis has shown unprecedented results in patients with MIBC [14], leading to a breakthrough therapy designation by the Food and Drug Administration. PD-L1 is usually considered as expressed on tumor cells or antigen-presenting cells [15], providing an inhibitory signal in PD-1-expressing T cells. Our data show that T cells themselves may be an important non-classical source of PD-L1 during BCG therapy. Importantly, tumor tissue immunohistochemical stainings revealed that the level of PD-L1 expression on immune cells, but surprisingly not on tumor cells, was associated with clinical response to PD-L1 blockade in MIBC patients [14]. However, it was not determined to which immune cell subset(s) PD-L1 expression was related. In light of our data, it would be of interest to better describe the profile of PD-L1-expressing immune cells in future studies investigating predictors of response to PD-L1/PD-1 blockade. However, such studies would be difficult to perform in urine samples recovered outside the BCG treatment course, as most samples do not show sufficient number of leukocytes to allow Tregs identification (Supplementary Fig. 7).

Altogether, our data provide additional arguments in favor of immunotherapeutic strategies combining BCG therapy with checkpoint blockade targeting the PD-1/PD-1 axis (NCT02324582 and NCT02792192 clinical trials) for the crucial need of improved treatments for high-risk bladder cancer patients.

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control condition. (E) Representative example of PD-L1+ Tregs analysis following the co-cultures; and (F) cumulative data from nine donors; (G) PBMCs from three healthy donors (left graph) or non-tumor (UROtsa) and tumor (Bu68.8, T24, UMC3) urothelium cell lines (right graph) were stimulated with BCG for 5 h, supernatants were harvested and IFN-β levels were measured by ELISA; (H) the latter supernatants from unstimulated or BCG-stimulated cell lines were also added to cultures of PBMCs from seven healthy donors, and PD-L1+ Tregs levels were then measured after 4 d; (I) PBMCs from four healthy donors were stimulated by indicated BCG-stimulated bladder cell supernatants in the presence of anti-IFN-β blocking antibody or an isotype IgG1 control; (J) an “urine immunosuppressive (IS) score” was calculated to take into account both cTregs and PD-L1+ Tregs levels during BCG therapy (mean of post-BCG samples from all patients). IS score is shown in 16 patients with different tumor stages and recurrence-free survival (Kaplan-Meier approach) is shown in patients with high versus low IS score (month 0 = first instillation; censored patients are represented by symbols). BCG = Bacillus Calmette-Guerin; cTregs = conventional regulatory T cells; HD = healthy donor; IFN = interferon; IS = immunosuppressive; MIBC = muscle-invasive bladder cancer; NMIBC = non-muscle invasive bladder cancer; PBMCs = peripheral blood mononuclear cells; UCa = urothelial cancer.
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Appendix A. Supplementary data

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References