ILC2-modulated T cell–to-MDSC balance is associated with bladder cancer recurrence

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Non-muscle–invasive bladder cancer (NMIBC) is a highly recurrent tumor despite intravesical immunotherapy instillation with the bacillus Calmette-Guérin (BCG) vaccine. In a prospective longitudinal study, we took advantage of BCG instillations, which increase local immune infiltration, to characterize immune cell populations in the urine of patients with NMIBC as a surrogate for the bladder tumor microenvironment. We observed an infiltration of neutrophils, T cells, monocytes, and monocytes to T cells and M-MDSCs, and group 2 innate lymphoid cells (ILC2). Notably, patients with a T cell–to-MDS ratio of less than 1 showed dramatically lower recurrence-free survival than did patients with a ratio of greater than 1. Analysis of early and late time points indicated that this patient dichotomy existed prior to BCG treatment. ILC2 frequency was associated with detectable IL-13 in the urine and correlated with the level of recruited M-MDSCs, which highly expressed IL-13 receptor α1. In vitro, ILC2 were increased and potently expressed IL-13 in the presence of BCG or tumor cells. IL-13 induced the preferential recruitment and suppressive function of monocytes. Thus, the T cell–to-MDSC balance, associated with a skewing toward type 2 immunity, may predict bladder tumor recurrence and influence the mortality of patients with muscle-invasive cancer. Moreover, these results underline the ILC2/IL-13 axis as a targetable pathway to curtail the M-MDSC compartment and improve bladder cancer treatment.

Introduction

Interactions between cancer and immunity have been intensively studied during the past 2 decades (1, 2). However, mechanisms hindering efficient constitutive and/or treatment-induced immune responses to human tumors have yet to be closely scrutinized. With rising incidence rates, bladder cancer (BCa) represents the fourth most commonly diagnosed cancer in men in Europe and the United States (3). Notably, BCa is the most expensive malignancy to treat per patient (4), mostly on account of dramatically high recurrence rates requiring lifelong follow-up with multiple surgical procedures. Most patients (>70%) present with a non-muscle–invasive BCa (NMIBC) at diagnosis, i.e., cancer that is confined to the mucosa (stage Ta, carcinoma in situ [CIS]) or submucosa (stage T1) (5). A major aim in the management of urothelial carcinoma is the prevention of the recurrence and progression of disease. For patients with an intermediate to high risk of recurrence, the standard treatment consists of intravesical instillations with the bacillus Calmette-Guérin (BCG) to prevent or delay tumor recurrence and progression (5). However, 20% to 30% of patients experience early recurrence, despite BCG treatment, and eventually undergo surgical removal of the bladder (5, 6). Several decades after this empirical immunotherapy was approved by the US FDA (7), the mode of action of intravesical BCG therapy remains unclear (8). Yet, it was shown that the strong inflammation induced locally encompasses (a) secretion of various chemokines and cytokines (9) and (b) infiltration of innate and adaptive immune cells, mostly neutrophils, T cells, and monocytes (8). This highlights the crucial need to better understand the regulation of immune responses that occur during the treatment of patients with NMIBC in order to identify new targetable pathways to improve BCa therapy.

In this prospective study, we performed a comprehensive analysis of immune cells in the urine of patients during BCG therapy, which increases the immune cell content locally. We found that the ratio of T cells to monocytic myeloid-derived suppressor cells (M-MDSCs), known to dampen antitumor immunity (10, 11), is strongly associated with recurrence-free survival outcomes. Of note, BCG treatment does not seem to alter this ratio. We also identified, for the first time to our knowledge in a human cancer, a putative role for group 2 innate lymphoid cells (ILC2), which were recently described as the innate counterpart of Th2 cells (12, 13). ILC2 levels correlated with M-MDSC levels in urine upon treatment with BCG. In vitro, bladder tumor cells and BCG were able...
to shift ILCs toward the ILC2 phenotype. Moreover, in response to BCG, ILC2 produced the type 2 cytokine IL-13, which mediated to shift ILCs toward the ILC2 phenotype. Moreover, in response to BCG, ILC2 produced the type 2 cytokine IL-13, which mediated to shift ILCs toward the ILC2 phenotype. Moreover, in response to BCG, ILC2 produced the type 2 cytokine IL-13, which medi- to shift ILCs toward the ILC2 phenotype. Moreover, in response to BCG, ILC2 produced the type 2 cytokine IL-13, which mediation of HLA–antigen D–related (HLA-DR) on monocytes, leading to an increase in the frequency of cells with an M-MDSC phenotype.

Results
Characterization of immune cells present in the urine during BCG therapy. An easy means for monitoring immune events in the bladder is to examine immune cells present in urine collected shortly after immunotherapeutic interventions. Thus, we performed a comprehensive phenotypic analysis of immune cells in urine samples from 28 patients with NMIBC during a standard 6-week intravesical BCG therapy. The patients’ characteristics are described in Table 1. Urine samples were obtained before and 4 hours after each instillation. As expected, most of the recruited cells were neutrophils (Figure 1A and B), but we also detected T cells (Figure 1A and C) (with variable CD4/CD8 ratios between samples and patients; data not shown), as well as monocytic cells (Figure 1D). Interestingly, a substantial number of urine CD14+ cells showed an M-MDSC phenotype (i.e., CD15 CD14+CD11b+CD33+HLA-DR+) (10, 11). These cells

Table 1. Characteristics of NMIBC patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients</th>
<th>Low T cell/MDSC ratio</th>
<th>High T cell/MDSC ratio</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>No. of patients</td>
<td>28</td>
<td>14</td>
<td>14</td>
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</tr>
<tr>
<td>Age, yr, median (IQR)</td>
<td>68 (62–74)</td>
<td>68.5 (63–80)</td>
<td>67 (60–73)</td>
<td>NS (P = 0.394)a</td>
</tr>
<tr>
<td>Sex, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Previous bladder tumor history, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No previous TURB</td>
<td>18 (64.3)</td>
<td>8 (57.1)</td>
<td>10 (71.4)</td>
<td></td>
</tr>
<tr>
<td>≥1 previous TURB</td>
<td>6 (21.4)</td>
<td>4 (28.6)</td>
<td>2 (14.3)</td>
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</tr>
<tr>
<td>≥2 previous TURB</td>
<td>4 (14.3)</td>
<td>2 (14.3)</td>
<td>2 (14.3)</td>
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</tr>
<tr>
<td>No. of second TURB, n (%)</td>
<td>23 (82.1)</td>
<td>11 (78.6)</td>
<td>11 (78.6)</td>
<td>NS (P = 1.0)b</td>
</tr>
<tr>
<td>No. of positive second TURB, n (%)</td>
<td>12 (42.9)</td>
<td>7 (63.6)</td>
<td>4 (33.3)</td>
<td>NS (P = 0.395)c</td>
</tr>
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<td>Tumor status, n (%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTa</td>
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<td>2 (14.3)</td>
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<tr>
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<td>1 (7.1)</td>
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<tr>
<td>Any CIS-positive</td>
<td>17 (60.7)</td>
<td>8 (57.1)</td>
<td>9 (64.3)</td>
<td>NS (P = 1)c</td>
</tr>
<tr>
<td>Grade, n (%)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>G2 low grade</td>
<td>3 (10.7)</td>
<td>1 (7.1)</td>
<td>2 (14.3)</td>
<td>NS (P = 0.487)c</td>
</tr>
<tr>
<td>G2 high grade</td>
<td>11 (39.3)</td>
<td>7 (50)</td>
<td>4 (28.6)</td>
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</tr>
<tr>
<td>G3</td>
<td>14 (50)</td>
<td>6 (42.9)</td>
<td>8 (57.1)</td>
<td></td>
</tr>
<tr>
<td>No. of tumors</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>5 (35.7)</td>
<td>5 (35.7)</td>
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</tr>
<tr>
<td>≥3</td>
<td>6 (21.4)</td>
<td>4 (28.6)</td>
<td>2 (14.3)</td>
<td></td>
</tr>
</tbody>
</table>

aUnpaired Student’s t test; bFisher’s exact test; cχ2 test; dThis patient was diagnosed with MIBC but refused cystectomy and instead received BCG therapy. IQR, interquartile range.
Although we observed trends for T cells and M-MDSCs, as the balance between effector and regulatory cells may be more relevant (17, 18), we then assessed for each patient the mean T cell/MDSC ratio in post-BCG urine samples. We segregated the patients into 2 groups: (a) patients with higher frequencies of M-MDSCs than T cells (i.e., log ratio <0, $n = 14$) and (b) patients with higher frequencies of T cells than M-MDSCs (i.e., log ratio >0, $n = 14$) (Figure 2D).

Of note, the ratio measured at the earliest time point available (i.e., pre-BCG 1 or 2) correlated with ratios obtained at later time points (the mean of all post-BCG samples) from the same patients (Figure 2E), suggesting that the patient dichotomy based on the post-BCG T cell/MDSC ratios already existed before BCG treatment initiation and arguing in favor of a prognostic role of the T cell/MDSC ratio. Notably, we found that the 2 groups strongly differed although we observed trends for T cells and M-MDSCs. As the balance between effector and regulatory cells may be more relevant (17, 18), we then assessed for each patient the mean T cell/MDSC ratio in post-BCG urine samples. We segregated the patients into 2 groups: (a) patients with higher frequencies of M-MDSCs than T cells (i.e., log ratio <0, $n = 14$) and (b) patients with higher frequencies of T cells than M-MDSCs (i.e., log ratio >0, $n = 14$) (Figure 2D).

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Figure 2. Recurrence-free and progression-free survival in patient groups following BCG therapy for NMIBC. Recurrence-free survival was assessed using the Kaplan-Meier approach in a cohort of 28 patients receiving BCG therapy (with the first instillation at month 0 and the last at month 1.5). Censored patients are represented by symbols. Patients were segregated into 2 groups \( (n=14\) patients in each group) on the basis of urine levels of neutrophils (A), T cells (B), or M-MDSCs (C), i.e., the mean percentage of CD15+, the mean percentage of CD3+, or the mean percentage of Lin-CD14-CD11b-CD33-HLA-DR+ cells above the median (High) versus below the median (Low) in post-BCG samples (2–6 time points per patient). (D) The mean T cell/M-DSC ratio in post-BCG urine samples identified 2 groups: patients with higher frequencies of M-MDSCs than of T cells (i.e., log ratio <0) and conversely (i.e., log ratio >0). (E) The T cell/M-DSC ratio was measurable at early time points before BCG (1 or 2) for 10 patients and correlated with the mean T cell/M-DSC ratios in post-BCG samples from corresponding patients. The line indicates linear regression, and Spearman’s rank correlation coefficients (R) and the corresponding P value are indicated. Recurrence-free survival (F) and progression-free survival (G) according to the T cell/M-DSC ratio. P values from log-rank tests are indicated on each panel. T, T cell.
CD4 T cells were analyzed for expression of Th1 and Th2 markers as well as for cytokine secretion profiles. While the proportions of CRTH2+ CD4 T cells were low in urine samples, they were found at higher levels in patients from the T/Mlo group (Supplemental Figure 4A). As expected, we detected high levels of the Th1 cytokines IFN-γ, IL-2, and TNF-α and low levels of the Th2 cytokines IL-4 and IL-5 in the supernatants of urine CD4+ T cells following 24-hour anti-CD3/CD28 stimulation (data not shown). Notably, patients in the T/Mlo group showed a higher Th2 to Th1 cytokine score (Supplemental Figure 4B). Moreover, since the Th1/Th2 balance may be regulated by tryptophan (Trp) metabolism (19, 20), we assessed local indoleamine 2,3 dioxygenase (IDO) activity by measuring the kynurenine/Trp ratio in pre- and post-BCG urine samples (at the fifth instillation). Accordingly, we found that the in terms of recurrence rates (log rank: P < 0.0001), as the median recurrence-free survival time following the start of BCG treatment was only 12.7 months in patients with a low T cell/MDSC ratio (T/Mlo), while it was not reached in patients with a high T cell/MDSC ratio (T/Mhi) (median follow-up of 32.7 months in this group) (Figure 2F). We also assessed the time to progression (Supplemental Figure 3B). Five patients from the T/Mlo group showed tumor progression versus one patient in the other group (Figure 2G).

Figure 3. Skewing toward ILC2 is associated with low T cell/MDSC ratios during BCG therapy for NMIBC. (A) Representative example of ILC labeling. Cells were gated on live lineage-negative CD127+ leukocytes (with lineage including CD3, CD14, CD56, CD19, CD11c, CD303a, CD15, CD203c, and FcεR1a). (B) Quantification of ILC1 (Lin CD127+ CRTH2– c-Kit–), ILC2 (Lin CD127+ CRTH2+), and ILC3 (Lin CD127+ CRTH2– c-Kit+) among urine cells (n = 23 samples). (C) Absolute number of ILC2 in post-BCG samples. (D) Cocultures of PBMCs from HDs (n = 6) with the NMIBC cell line Bu68.8, BCG (MOI = 1), or medium alone for 4 days. Repartition of ILC1, ILC2 and ILC3 subsets as well as frequencies of total ILC are shown for each indicated conditions. (E) ILC2 frequencies in post-BCG urine samples from patients with high (T/Mhi, n = 10) or low (T/Mlo, n = 13) T cell/MDSC ratios, as identified in Figure 2, were compared (2-tailed Student’s t test). ILC2 frequencies were correlated with the T cell/MDSC ratios (F) as well as T cell (G) and M-MDSC (H) frequencies in corresponding urine samples. Lines indicate linear regression. Spearman’s rank correlation coefficients (R) and the corresponding P values are indicated in F–H. *P < 0.05, **P < 0.01, and ****P < 0.0001, by 1-way ANOVA followed by Tukey’s test (B), a post test for linear trend (C), or Dunnett’s test (D) for comparison of subsets, longitudinal changes, or conditions versus medium, respectively.
level of IDO activity was higher in samples from patients in the T/Mlo group (Supplemental Figure 4C).

We next analyzed the presence and repartition of ILCs in urine samples from patients during their BCG therapy. While the levels of type 1 and type 3 ILCs were low, we found measurable levels of ILC2 (Lin–CD127+CRTH2+ cells) in the urine (Figure 3, A–C). In consonance with this observation, in vitro experiments using PBMCs from HDs (Figure 3D) showed that BCa cells and BCG were able to significantly augment ILC2 proportions among total ILCs, at the expense of ILC3 and ILC1, respectively. Moreover, only BCG tended to induce an increase in total ILC frequencies (Figure 3D).

Of note, in contrast to Ty21a, heat-killed BCG also significantly increased the percentage of ILC2, albeit to a lesser extent compared with the percentage of ILC2 with live BCG (Supplemental Figure 5).

We also sought evidence to explain how ILC2 could infiltrate the bladder in this context. It was recently reported that ILC2 expressed the chemokine receptor CCR4 and did so at higher proportions than was observed with ILC1 or ILC3 (21). We confirmed that CCR4 was expressed on peripheral ILC2 from patients with NMIBC, and at a significantly higher frequency than was observed on ILC1 or ILC3 (Supplemental Figure 6A). Notably, ligands for CCR4 (macrophage inflammatory protein 1α [MIP-1α], monocyte chemoattractant protein 1 [MCP-1], regulated on activation, normal T cell expressed and secreted [RANTES, also known as CCL5], and CCL22 [also known as MDC]) have been reported to be induced locally during BCG therapy (9, 22–25). Of note, we were able to measure CCR4 expression in ILCs from 3 urine samples during BCG therapy (Supplemental Figure 6B) and found that most ILC2 but also, in contrast to the periphery, most ILC1 and ILC3 expressed CCR4 in the urine. Altogether, these data suggest that CCR4 may be involved in the recruitment of ILCs in the bladder during BCG therapy, with a prefferential enrichment in ILC2, since ILC2, compared with ILC1 and ILC3, express a higher proportion of CCR4 in the periphery. Besides, bladder tumor cells are able to produce low levels of prostaglandin D2 (PGD2) (Supplemental Figure 6C), a chemoattractant for ILC2 (26), which could also contribute to the recruitment of ILC2 in the bladder, as shown in the inflamed lung (27).

Notably, patients in the T/Mlo group showed higher levels of ILC2 in the urine (Figure 3E). The negative correlation between the T cell/MDSC ratio and ILC2 levels (Figure 3F) is probably explained by a putative link between ILC2 and M-MDSCs, as ILC2 levels positively correlated with M-MDSCs but not with T cells (Figure 3, G and H). Altogether, these results suggest that local type 2 immunity skewing is linked to the immune profile associated with BCG treatment failure.

We next evaluated ILC2 and M-MDSCs in blood from the same patients (i.e., BCG-treated NMIBCs) but also from patients with more advanced (i.e., muscle-invasive BCa [MIBC]) at the time of cystectomy (Supplemental Table 1). As compared with HDs, patients with NMIBC had normal levels of circulating M-MDSCs and ILC2 (Figure 4, A and B), with no difference between the T/MMhi and T/MMlo groups (data not shown). In contrast, patients with MIBC showed increased peripheral blood levels of both cell subsets (Figure 4, A and B). Of note, the proportion of ILC2, but not ILC1 or ILC3, was positively correlated with M-MDSC levels in the blood of MIBC patients (Figure 4C), which substantiates the observations in urine.
Potential involvement of an ILC2/IL-13/MDCS axis in BCG failure. We tested the presence of ILC2-associated cytokines in urine samples. While IL-4 and IL-5 were undetectable (data not shown), IL-13 was detected in 6 of 13 post-BCG samples corresponding to patients with higher frequencies of ILC2 in the urine as compared with those with IL-13 levels below the detection limit (Figure 5A). We then performed an in vitro evaluation of the propensity of ILC2 to produce IL-13 in the context of BCG stimulation. PBMCs from both HDs and patients with MIBC, who showed elevated ILC2 levels (Figure 4B), were stimulated with BCG for 48 hours, and we then measured IL-13 production in ILC2 (Figure 5B). Interestingly, ILC2 were strongly able to express IL-13 in response to BCG, with higher proportions found in ILC2 from MIBC patients compared with ILC2 from HDs (Figure 5C). Besides, in contrast to nonmalignant urothelium (HCV-29 cell line) and to the Bu68.8 cell line, the MIBC cell line (TCC-Sup) significantly upregulated IL-13 expression in ILC2 from patients with MIBC (Figure 5D).

Hence, we next investigated the potential contribution of an ILC2/IL-13-mediated mechanism in M-MDCS recruitment and/or induction. While IL-13 is known to alternatively activate macrophages (M2) (28), the role of IL-13 in MDSC biology in humans remains elusive. To consider a potential effect of IL-13 on human M-MDSCs, we first examined whether these cells express the IL-13 receptor α1 (IL-13Rα1) (Figure 5E), as was recently suggested in a mouse study (29). As shown in Figure 5F, monocytes from both HDs and patients expressed IL-13Rα1. We observed a higher receptor density (a) on M-MDSCs compared with monocytes and (b) in urine compared with circulating M-MDSCs. We then investigated the role of IL-13 on monocytic cells. First, we found in a Transwell experiment using PBMCs from HDs that IL-13 was able to preferentially attract monocytes in contrast to lymphocytes (Figure 5G), as suggested in a previous study (30). We next functionally assessed whether IL-13 may confer suppressive activity to monocytic cells. Therefore, blood monocytes isolated from HDs were cultured in the presence of granulocyte macrophage-CSF (GM-CSF) (as a survival factor) for 4 days, with or without recombinant IL-13. Cells were subsequently used in autologous T cell proliferation assays. Analysis of CFSE dilution in cocultured T cells stimulated by anti-CD3/CD28 showed that IL-13–treated CD14+ cells were able to significantly inhibit the proliferation of both CD4+ and CD8+ T cells, in contrast to untreated CD14+ cells (Figure 5H–J). Phenotypic analysis of the IL-13–treated monocytic cells is shown in Supplemental Figure 7. Notably, IL-13 downregulated HLA-DR expression, thereby increasing the frequencies of HLA-DRα cells in sorted CD14+ cells (Supplemental Figure 7, A and B). Of note, while CD115 (CSF1-R) was upregulated following IL-13 treatment in both HLA-DRα and HLA-DRβ cells, all other monocyte and macrophage surface markers tested showed lower expression, including that of the M2 marker CD163 (Supplemental Figure 7C). Therefore, it seems that the ability of IL-13 to induce suppressive function in monocytic cells is attributable to an induction of M-MDSCs rather than of M2 macrophages, although the differences between these subsets remain unclear (11). Furthermore, IL-13 transcriptionally upregulated ARG1, iNOS, and C/EBPβ (Supplemental Figure 7D), which were also found to be expressed in urine M-MDSCs from patients (Figure 1, D and E). Moreover, IL-13 treatment of CD14+ cells increased the secretion of IL-1 receptor antagonist (IL-1RA), an anti-inflammatory molecule blocking IL-1 signaling, while it decreased the secretion of MCP-1 and IL-8, a neutrophil-recruiting cytokine known to be associated with successful BCG therapy in patients with BCa (31) (Supplemental Figure 7E).

To substantiate an IL-13–mediated mechanism in M-MDSC induction upon BCG treatment, we tested the effect of BCG-stimulated PBMC supernatant on monocytic cells in the absence or presence of an anti–IL-13–blocking antibody. HD PBMCs were stimulated with BCG for 3 days, and supernatants were harvested. As shown in Figure 5K, IL-13 was induced upon BCG stimulation. Filtered supernatants were then added onto sorted CD14+ cells in the presence of either an anti–IL-13–blocking antibody or an IgG1 isotype control. After 4 days, cells were used in a T cell proliferation assay as above. Interestingly, we found that T cell proliferation, which was lower in the presence of monocytic cells conditioned by BCG-treated versus untreated supernatants, was partially recovered when IL-13 was blocked (Figure 5, L and M).

Analyses of overall survival data from TCGA. We performed a large-scale analysis of bladder urothelial cancer data from TCGA (32). In the absence of TCGA data from patients with NMIBC, this analysis confirmed our findings and extended them to patients with MIBC. These data included tumor mRNA expression and survival data from 300 patients with advanced-stage tumors (T2–T4 for >98% of samples). We observed a counterbalancing effect of monocytic cells on T cells (appraised on the basis of CD14 and CD3E gene expression, respectively) (Figure 6A). Accordingly, short survival was associated with “low CD3, high CD14” expression and long survival with “high CD3, low CD14” expression, while intermediate survival was observed in the scenarios of “low CD3, low CD14” and “high CD3, high CD14” expression (Figure 6B). Moreover, the association analysis of CD14 and IL-13 expression corroborated a link between the presence of IL-13 in the tumor and a recruitment of CD14-expressing cells (Figure 6C). Finally, a counterbalancing effect holds between T cells and the presence or absence of IL-13 in the tumor, as the shortest survival was found in patients with low T cell infiltration, together with detectable IL-13 (Figure 6D).

While CD3 expression accurately defines T cells, 1 single marker (CD14) is not sufficient to identify M-MDSCs, so that the influence of M-MDSCs cannot be fully deconvoluted in TCGA data. Also, one cannot exclude the possibility that IL-13 expression also captures other subtypes of immune cells that may play a role in patient survival. Nevertheless, these in silico analyses are consistent with our experimental results and suggest that the mechanisms uncovered in this study may broadly apply to both NMIBC and MIBC.

Discussion

In this prospective, longitudinal study of patients with BCa, we observed a gradual increase in the absolute numbers of all immune cell subsets tested in urine during the course of BCG immunotherapy. In contrast to individual cell subsets, the T cell–to–MDSC ratio, likely preestablished before treatment, was associated with the clinical outcome, showing that the balance between these cell subsets may be critically involved in tumor recurrence, when tilted toward the M-MDSC side (Figure 7). For the first time to our knowledge in humans, we also identified ILC2 as new players in
Figure 5. IL-13 is produced by ILC2 upon BCG stimulation and is able to recruit and induce suppressive function in monocytic cells. (A) Frequencies of ILC2 in urine samples with undetectable ($n=7$) versus detectable ($n=6$) urinary IL-13 (detection limit: 5 pg/ml). (B) Representative FACS histograms of IL-13–producing ILC2 upon stimulation with BCG (MOI = 1) or medium alone in PBMCs from HDs ($n=6$) and MIBC patients ($n=5$) and (C) their quantification. (D) IL-13 production in ILC2 from patients with MIBC following coculture with nonmalignant urothelium (HCV-29), Bu68.8, or TCC-Sup tumor cell lines. (C and D) Statistical analysis was performed using a 2-tailed, paired Student’s $t$ test to compare stimulated versus medium-only conditions and a 2-tailed, unpaired Student’s $t$ test to compare patients with HDs (C) and 1-way ANOVA followed by Dunnett’s test to compare cell lines with the control condition (D). (E) Representative example of IL-13R$\alpha$1 labeling. (F) Levels of ex vivo IL-13R$\alpha$1 expression on the indicated monocytic cells from PBMCs obtained from HDs ($n=9$), NMIBC patients ($n=18$), and MIBC patients ($n=10$) and from post-BCG urine samples ($n=8$), expressed as the ratio of mean fluorescence intensity (RFI) of specific staining versus the isotype Ig control. A 2-tailed, paired Student’s $t$ test was performed to compare M-MDSCs with CD14$^+$HLA-DR$^{+}$hi cells and an unpaired Student’s $t$ test to compare urine with circulating counterparts. (G) Chemotactic response of CD14$^+$ cells and CD3$^+$CD19$^+$ lymphocytes from HD PBMCs ($n=6$; upper chamber) to medium alone or IL-13 (100 ng/ml; lower chamber) using Transwell plates. (H) Representative FACS histograms of CFSE-labeled activated T cells cultured alone or with purified CD14$^+$ cells pretreated with IL-13 (IL-13–treated CD14$^+$ cells) or not (CD14$^+$ cells). (I and J) Quantification of the proliferation index in CD8$^+$ (I) and CD4$^+$ (J) T cells. (K) IL-13 concentration in supernatants of PBMCs ($n=8$) subjected to 3 days of stimulation with BCG or no stimulation. (L) Representative example of CFSE-labeled activated CD8$^+$ T cells cocultured with CD14$^+$ cells previously conditioned with filtered supernatants of unstimulated or BCG-stimulated PBMCs in the presence of either anti–IL-13–blocking antibody or isotype IgG1 control (10 $\mu$g/ml each). (M) Comparison of the proliferation index in each indicated condition ($n=3$, representative of 2 independent sets of experiments). *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$, by 2-tailed, paired Student’s $t$ test (G–K) and 1-way ANOVA followed by Tukey’s test (M). Div, percentage of cells with at least 1 division; Ig, isotype IgG1 control; PI, proliferation index; SN, supernatant.
While MDSCs have been extensively studied in mouse cancer models (10), the data are sparser with regard to their clinical prognostic value in human cancers (34). Most studies consistently found — mainly in peripheral blood — elevated granulocytic and/or monocytic MDSC levels in patients with various cancers, including BCa (35, 36), and the levels were often associated with the tumor stage (34), which is in line with our peripheral MDSC data. In tissue, high infiltration of CD68+ tumor–associated macrophages (TAMs) was associated with a poor response to BCG immunotherapy (37). Notably, the beneficial versus deleterious role of the tumor-associated immune contexture (38) might depend on the localization of immune cell subsets, which can be associated with different clinical outcomes according to whether these subsets are assessed in the tumor core versus the invasive margin, or inside versus outside the tertiary lymphoid structures (39). Likewise, the level of CD68+ macrophages in bladder CIS was associated with a poor response to BCG therapy only when measured in the tumor, and not in the bladder lamina propria (40). However MDSCs, in contrast to T cells, cannot be accurately appraised in situ by IHC. This points out the unmet need to identify specific and sensitive markers able to reliably define MDSCs for in situ assessment.

The growing field of ILC research is still in its infancy for human cancers (41, 42), although some recent studies reported on ILCs in murine tumor models. ILC1, and potentially ILC3, may be involved in tumor immunosurveillance (43, 44). However, the role of ILC2 — otherwise involved in anti-helminth immunity and tis-
Both tumor cells and BCG may promote the ILC2/IL-13/MDC axis. We showed that BCa cells are able to induce suppressive M-MDSCs in vitro, as was previously observed in different cell lines from various cancers (54). Thus, a basal immunosuppressive level may exist in the bladder before treatment, as supported by the correlation between early pre-BCG and late post-BCG samples. Second, intravesical BCG stimulation triggers a substantial immune infiltration, including infiltration of M-MDSCs and ILC2, the composition of which may be the result of different events: modulation/proliferation of already locally present immune cells, recruitment from the periphery by urothelium-derived chemokines, and, in a second phase, modulation of the locally recruited cells. While mycobacteria are potent inducers of T(h)1 responses (55), which are required for BCG-mediated bladder tumor control (8), we found that BCG can also induce MDSCs, as shown in intradermally BCG-vaccinated mice (56). In addition, BCG shifted ILCs toward the ILC2 phenotype, at the expense of ILC1. A recent study supports this finding, reporting an increase in ILC2 levels in Mycobacterium leprae/HIV–coinfected patients compared with levels in HIV-monoinfected and healthy individuals (57). Further investigations may evaluate whether ILC2 skewing is due to ILC2 proliferation or repolarization from another ILC subset, as was recently suggested (58, 59).

While induction of ILC2 following mycobacteria infection/instillation may drive tissue repair (12, 13), these cells may be also involved in the pathophysiology of diseases such as cancer, in which type 1 immunity is required. In BCa, besides inducing a strong T(h)1 response, which may depend on preexisting BCG-specific adaptive immunity (60), BCG may also amplify a pre-established immunosuppressive tumor contexture containing MDSCs and ILC2. This needs to be compensated by a higher BCG-induced T cell infiltration, without which patients experience rapid tumor recurrence.

While immunotherapy using an anti–PD-L1 antibody was recently found to show clinical efficacy in advanced metastatic BCa, with a breakthrough status designated by the FDA (61), new combination treatments are urgently needed for NMIBC to improve BCG therapy and lower BCG failure rates (62). This study highlights the crucial need to restrain the tumor-induced “immunosuppressive switch” (63) by targeting MDSCs to tilt the balance in favor of T cells. A number of compounds were found to target MDSCs (10, 11). One of these compounds, doxorubicin, may be of interest, as it selectively targets monotypic MDSCs in humans (64). However, in light of our results, all-trans retinoic acid (ATRA, also known as vitamin A) may be an ideal candidate treatment on the basis of the following properties: (a) this compound is also known to eliminate MDSCs and thus to augment antigen-specific T cell responses (65), including in cancer patients (66); (b) Spencer et al. recently reported that vitamin A strongly restrains ILC2 levels, particularly...
ILC2 that produce IL-13 (67); and (c) a meta-analysis showed that vitamin A plays a protective role in BCa (68), and ATRA treatment was found to significantly improve survival and recurrence rates in NMIBC patients (69). Furthermore, a combination of depletion or attenuation of MDCs and concomitant stimulation of effector T cells may be of interest. In this regard, programmed death 1 (PD-1) and cytotoxic T lymphocyte–associated protein 4 (CTLA-4) combination blockade has proven to increase the T cell–to-MDSC ratio in melanoma tumors in mice (18).

The present study highlights the importance of the immune microenvironment in the clinical outcome of BCG-treated superficial BCa and suggests a possible new role for ILC2 in human cancer. We propose that the ILC2/IL-13 axis is a mechanism driving an immunosuppressive microenvironment that is critically involved in the failure of BCG immunotherapy (Figure 7). Such a microenvironment could also influence the mortality of patients with more advanced cancer, as suggested by our analysis of BCA data from TCGA. Altogether, these results imply that M-MDCs and novel pathways, such as IL-13/IL-13R signaling or ILC polarization, may represent attractive targets for potentiating cancer immunotherapies.

Methods

Study populations. Twenty-eight patients with NMIBC were recruited in this prospective, longitudinal study at the University of Lausanne Hospital. At the time of inclusion, patients started standard BCG therapy involving 6 weekly intravesical instillations of TICE BCG (MD: Merck Sharp & Dohme AG) (2 × 10^8 to 8 × 10^8 CFU), according to European Association of Urology guidelines and to the decision of both physicians and patients. NMIBC patients’ characteristics are described in Table 1. Urine samples were obtained before and 4 hours (± 1 hour) after each instillation. Peripheral blood was also collected at baseline and during week 4. In addition, peripheral blood from 18 patients with MIBC was collected at the time of cystectomy. MIBC patients’ characteristics are described in Supplemental Table 1. Blood samples were also collected from healthy volunteers (>50 years of age) through the local Swiss blood bank.

Clinical follow-up. Following BCG therapy, flexible cystoscopy and cytology were performed on a regular basis. In instances of positive cytology results and/or a visible tumor, subsequent transurethral resection of the bladder (TURB) allowed confirmation of the presence or absence of a recurrent tumor. Tumor progression was defined as the recurrence of a tumor at a higher stage or grade and/or worsening of disease, as defined in refs. 6 and 70.

Flow cytometric analysis of urine-infiltrating immune cells. Urine samples were centrifuged to separate cells from the urine supernatant. The total number of cells in urine was independent of the voided volume (our unpublished observations). The following mAbs were used at predetermined optimal concentrations: anti-CD45-phycocerythrin (PE) (2D1); anti–CD11b–allophycocyanin (APC)/eFlour 780 (ICRF44); anti–CD3–PerCP/Cy5.5 (SK7); anti–FcγRIIa–FITC (AER-37, CRLA1); anti–CD3–PE/Cy7 (UCHT1); anti–CD14–PE/Cy7 (6D3); anti–CD19–PE/Cy7 (SJ25Cl); anti–CD303a–PE/Cy7 (201A) (all from eBioscience); anti–CD14–Pacific Blue (HCD14); anti–CD15–PerCP/Cy5.5 (W6D3); anti–CD3–APC (WM53); anti–HLA-DR–PE/Cy7 (L243); anti–CD15–Pacific Blue (W6D3); anti–CD56–PE/Cy7 (HCD56); anti–CD117 (c-Kit)–PE (1D4D2); anti–CD294 (CRTH2)–PerCP/Cy5.5 (BM16); anti–CD11c–PE/Cy7 (Bu15); anti–CD15–PE/Cy7 (W6D3); anti–CD163–PE (GHI/61); anti–CD64–PerCP/Cy5.5 (10.1); and anti–CD68–AF647 (Y1/82A) (all from BioLegend); anti–CD3–PE/AF610 (7D6); anti–CD19–PE/AF610 (SJ25C1); anti–CD56–PE/Texas Red (MEM-188); and anti–CD8–PE/AF610 (B85) (all from Invitrogen, Thermo Fisher Scientific); anti–CD4–APC/H7 (RPA-T4); anti–CD127–AF647 (HIL-7R-M21); anti–CD203c–BV421 (NP4D6); anti–CD80–APC/H7 (L307); anti–CD115 (CSF-1R)–BB515 (9-4D2); and anti–CCR4–PE/Cy7 (1G1) (all from BD Biosciences); and anti–IL-13Rα1–PE (41978N) (from R&D Systems).

Cells were stained for surface antigens for 20 minutes at 4°C, and an amine reactive dye (Aqua LIVE/DEAD Stain Kit; Life Technologies, Thermo Fisher Scientific) was used for dead cell exclusion according to the manufacturer’s instructions. Fc-Receptor Blocking Reagent (Miltenyi Biotec) was used to increase staining specificity by blocking unwanted binding of antibodies. Sample acquisition was performed on the Gallios Flow Cytometer (Beckton Coulter), and data were analyzed using FlowJo software.

Intracellular cytokine staining. Cells were stimulated with BCG (MOI = 1) for 48 hours, and 2.5 μg/ml brefeldin A was added for the last 16 hours of culture. Staining for cell-surface markers was carried out as described above, and cells were then fixed. Intracellular staining was performed with APC-conjugated anti–IL-13 mAb (JES10-5A2; BD Pharmingen) for 30 minutes at 4°C in 0.1% saponin (Sigma-Aldrich).

Cell lines and bacteria. Bu68.8 is a noninvasive BCA cell line isolated from a patient with a TaG2 tumor at the urology department of the University of Lausanne Hospital. T24 and TCC-Sup are invasive urothelium carcinoma cell lines corresponding to histological grades G3 and G4, respectively (71) (gift of G.N. Thalmann, Inselspital, Bern, Switzerland). HCV-29 is a nonmalignant cell line derived from normal urothelium (71) (gift of Anna Lityńska, Jagiellonian University, Kraków, Poland). For the cocultures, tumor cells were seeded to achieve confluence by the end of the experiment as determined beforehand (e.g., a ratio of approximately 1:20 with PBMCs in 4-day-long experiments).

Bacteria used for in vitro experiments were obtained by resuspension in PBS of the lyophilized content of clinically available oncoTICE BCG vials and Vivotif Ty21a capsules.

Suppression assays. Monocyte isolation was performed using the Dynabeads FlowComp Human CD14 Kit (Invitrogen) from cryopreserved PBMC or as previously described (72) from fresh blood (>85% purity). CD14+ cells were cultured for 4 days with 400 U/ml GM-CSF (ImmunoTools) in the presence or absence of 100 ng/ml recombinant human IL-13 (BioLegend), or with filtered supernatant (70 %) from PBMCs stimulated or not for 72 hours with BCG (MOI = 1). When indicated, anti–IL-13–neutralizing antibody or the corresponding IgG1 isotype control (10 μg/ml; BioLegend) was added. Autologous T cells were isolated from PBMCs using the Dynabeads FlowComp Human CD3 Kit (Invitrogen, Thermo Fisher Scientific), stained using 5 μM CFSE for 5 minutes at room temperature, and washed twice with ice-cold PBS containing 5% FCS. CD14+ cells were cocultured for 4 days with CFSE-labeled T cells at a 1:1 ratio in 96-well plates previously coated with 1 μg/ml anti–CD3 (OKT3) antibody and soluble anti–CD28 (Biolegend) (1 μg/ml) and IL-2 (150 U/ml; Roche). Proliferation of CFSE-labeled cells was assessed by flow cytometry after surface staining for CD3, CD4, and CD8. The proliferation index (total number of divisions divided by the number of cells that went into division) was assessed using the FlowJo Proliferation Platform.
Sorting of urine cell subsets, cDNA synthesis, and real-time qPCR. Lin CD14+CD33+HLA-DRlo and Lin CD14+CD33+HLA-DRhi cells were FACS sorted from post-BCG urine samples and subsequently tested for purity (Supplemental Figure I). Five samples from patients included in the present cohort as well as two samples from patients receiving SII-ONCO-BCG at the University Hospital of Basel were tested. Dry pellets (900–10,000 cells) were then resuspended in a lysis cDNA mix solution containing 6.3 μl lysis buffer (prepared as previously described by Gupta et al. [ref. 73]); 3 μl 5× reverse transcriptase (RT) buffer (73); 1.5 μl of 0.1 M DTT (AppliChem); 0.75 μl of 10 mM deoxyribonucleotide triphosphates (dNTPs) (Invitrogen, Thermo Fisher Scientific); 0.25 μl of 100 ng/μl oligo-d(T) (Metabion); 0.4 μl Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific); 0.2 μl RNasin (Promega); and 2.6. μl RNA-free water. To allow reverse transcription into cDNA, samples were incubated at 37°C for 60 minutes. The transcriptase was then inactivated at 90°C for 3 minutes, and samples were stored at −80°C. Quantitative real-time PCR was performed using KAPA SYBR FAST Master Mix with ROX (KAPA Biosystems), 200 nM of each primer, and one-twentieth of the reverse transcription reaction. Samples were run at least in duplicate in an ABI 7500 Fast Real-Time PCR Thermo Cycler (Applied Biosystems) with the following parameters: 3 minutes at 95°C for enzyme activation, 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds, followed by ampiclon melting analysis to evaluate the specificity of the reaction and identify the presence of primer dimers. B2-microglobulin (B2M) was used as the reference gene, as it was previously identified as one of the most stable genes in activated mononuclear cells (74). Primers were purchased from Microsynth, and efficiency was validated using cDNA serial dilutions. The primers sequences were as follows: B2M, 5′-CCAGCAGAGATGGAAG-3′ (forward) and 5′-GATGCTGTCATAGTGCT-3′ (reverse); ARG1: 5′-ATTCTTCCGTCTCCTGACTT-3′ (forward) and 5′-AGTGATGTAAGGTATTAG-3′ (reverse); INOS: 5′-ATGCTACGTCATCCGGCTAT-3′ (forward) and 5′-CACAGGTCAGGGTGAT-3′ (reverse); and CEBPB: 5′-GCGCGTTTGGAGGGTTG-3′ (forward) and 5′-CAACAGGGCGGTAAGACAT-3′ (reverse). Results are expressed using the 2−ΔΔCt method.

Measurement of soluble molecules. Cell-free urine samples were stored at −80°C. Cytokines were measured using a Luminex assay according to the manufacturer’s instructions (Thermo Fisher Scientific), and tryptophan and kynurenine levels were measured by Metabion Inc. using ultra-high-performance liquid chromatography–tandem mass spectroscopy (UPLC-MS/MS) (75). The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer. Peaks were quantified using the AUC, and relative kynurenine levels were divided by those of tryptophan to assess the K/T ratio in each sample.

Ex vivo–expanded urine T cells. Cells from urine samples were stimulated, either directly or following magnetic T cell enrichment, with PHA (1 μg/ml) and IL-2 (150 U/ml) in the presence of irradiated allogenic PBMCs for 2 to 4 weeks, as we previously described (76). When T cells successfully expanded, CD4+ T cells were isolated using the Dynabeads FlowComp Human CD4 Kit (Invitrogen, Thermo Fisher Scientific) and stained for Th1 and Th2 surface markers. The remaining CD4 cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 for 24 hours, and the supernatant was collected to measure IFN-γ, IL-2, TNF-α, IL-4, and IL-5 cytokines using a Cytometric Bead Array (BD Biosciences). For each sample, the concentration [i] of the cytokine i was normalized to the maximum concentration [i]max, observed for this cytokine (Equation 1), and the Th2 versus Th1 score (Equation 2) was determined. These equations were calculated as follows:

\[
C_i = \frac{[i]}{[i]_{\text{max}}} \times 100
\]

(Equation 1)

\[
\text{Score} = \frac{C_{\text{CD4}} - C_{\text{CD8}}}{3} + \frac{C_{\text{CD8}} - C_{\text{CD4}}}{3}
\]

(Equation 2)

Data and computational analysis in TCGA. Gene expression data for bladder urothelial cancer from TCGA (52) were obtained through release 18 from the International Cancer Genome Consortium (ICGC; https://icgc.org/). Gene expression was normalized to counts per million before analysis. Survival data for the same patients were obtained with the Firebrowse package in R (https://github.com/mariodeng/FirebrowseR). We used the R survival package to plot the Kaplan-Meier curves and estimate the log-rank difference between the various groups of donors studied. The analysis was done on the overall survival of the donors. As IL-13 was not detectable in all samples, we defined a threshold, assuming IL13 was present in the sample when more than 2 reads mapped to its gene.

Computation of excess CD14. The level of infiltration of the different immune cells is in general highly correlated, such that we could model at first approximation the expression of CD14 as:

\[
\text{CD14} = a \times \text{CD45} + β
\]

(Equation 3)

To study the effect of IL13 on CD14, it is important to estimate how much the expression of CD14 differs from the expected value coming from the global immune infiltrate. Using all the samples, we estimated the coefficients a and β from Equation 3. Then, for each patient, we computed the residue of this fit: a positive residue corresponded to an excess of CD14 with respect to the expected amount, while a negative value represented a deficiency in CD14. The residues are shown in Figure 6C.

Statistics. Comparisons between samples and individuals or between conditions in the same individuals were evaluated using an unpaired or paired (2-tailed) Student’s t test, respectively, directly or after log transformation to match normality assumptions. For multiple comparison analyses, 1-way ANOVA (for paired or unpaired values as appropriate) was performed, followed by Dunnett’s correction when all data were compared with a control condition, by Tukey’s correction when all data were compared, one with another, or by a post test for linear trend to assess longitudinal data. A P value of less than 0.05 was considered statistically significant. The Kaplan-Meier estimator was used to assess recurrence- and progression-free survival rates. The log-rank test was performed to analyze potential differences in survival rates between groups. The median follow-up time was calculated by using the reverse Kaplan-Meier method (77). Comparisons of patients’
characteristics were performed using a Fisher’s exact or χ² test. All statistical analyses were performed using R or GraphPad Prism, version 6 (GraphPad Software).

Study approval. This study was approved by the ethics committee of the canton of Vaud, Switzerland. Some urine samples were obtained from patients receiving SII-ONCO-BCG at the University Hospital of Basel with the approval of the Basel ethics committee (protocol EKBB161/09). All patients provided written informed consent prior to their participation in the study.

Author contributions

MFC, ST, CJ, and LD designed the study and wrote the manuscript. MFC, ST, BS, VC, D. Gharbi, PB, and SDP performed and analyzed the experiments. JR and D. Gfeller conducted in silico analyses. MFC, ST, JR, and LD performed statistical analyses. JR, DNH, CAR, DES, D. Gfeller, and PJ provided intellectual input and edited the manuscript. FD, ASF, CAR, and PJ recruited the study participants and provided patients’ samples and clinical data. CJ and LD jointly supervised the study.

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