Brief Correspondence

Siglec-6 as a New Potential Immune Checkpoint for Bladder Cancer Patients

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Abstract

Among the growing family of inhibitory receptors regulating immunity, sialic acid–binding immunoglobulin domain–containing lectins (Siglecs) have recently emerged as immunoregulatory receptors recognizing sialylated ligands on tumor cell surface. However, their role in the immunoregulation of bladder cancer (BCa) remains unknown. Here, we determined the presence of eight Siglec ligands (SLs) on bladder non-tumor and tumor cell lines. S2L, S3L, and S6L were not expressed, and few bladder tumor cell lines expressed S5L and S14L. In contrast, S7L and S10L were upregulated on all bladder tumor cell lines. We found a discrepancy in S9L expression by non-tumor cell lines, which is however highly expressed by bladder tumor cell lines. Notably, expression of S5L, S6L, and S14L was increased upon bacillus Calmette-Guérin (BCG) infection. Furthermore, we analyzed the expression of Siglecs on T cells from healthy donors and BCa patients. Circulating T cells only expressed Siglec-6, which is upregulated in non-muscle-invasive BCa patients. In addition, BCG therapy induced the overexpression of Siglec-6 by urinary CD8+ T cells. In vitro functional assays suggested that Siglecs may decrease cytotoxic functions of effector CD8+ T cells. Finally, analyses from two BCa datasets (The Cancer Genome Atlas and UROMOL cohorts) showed that Siglec-6 is associated with tumor progression and poor survival. Our findings indicate that Siglec-6 might be a new target for BCa treatments.

Patient summary: We investigated the expression of Siglecs, a family of immunoregulatory receptors, in bladder cancer patients. We observed that the expression of Siglec-6 is increased on circulating and urinary T cells of non-muscle-invasive bladder cancer patients. We also showed that Siglec-6 is associated with lower survival in bladder cancer patients and might contribute to bladder cancer recurrence.

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Bladder cancer (BCa) is a highly prevalent disease and associated with substantial morbidity, mortality, and cost per patient [1]. Although bacillus Calmette-Guérin (BCG) therapy is the gold-standard treatment for non–muscle-invasive bladder cancer (NMIBC) patients, this treatment is limited due to significant side effects, with discontinuation in 25% of patients owing to poor tolerance and failure [2]. This underlies the urgent need to improve current standard therapy and/or develop new treatments against BCa. Urothelial cancers developed a plethora of mechanisms to evade antitumor immunity, including the overexpression of inhibitory receptors and their ligands, known as immune checkpoints [3]. Therapeutic targeting of immune checkpoints in humans recently demonstrated the great potential of such an approach, especially in BCa [4]. Sialic acid-binding immunoglobulin domain-containing lectins (Siglecs) have recently emerged as receptors linked to immune regulation through the recognition of sialic acid-containing glycans [5]. Although several recent reports highlighted an immunoregulatory role for Siglecs in cancer patients [5–7], almost no data are available on the expression of Siglecs and their ligands in BCa. Siglecs are expressed on most of the immune cells [7,8]. In humans, at least 14 Siglecs have been identified. Except for Siglec-1, all Siglecs comprise structural motifs responsible for intracellular signaling. Most of Siglecs contain an immunoreceptor tyrosine-based inhibitory motif (ITIM), an immunoreceptor tyrosine-based switch motif, or an ITIM-like motif participating in an inhibitory signal. In contrast, Siglec-14, Siglec-15, and Siglec-16, which can associate with the coreceptor DAP12, are considered as activatory receptors [6,7]. Siglecs have the distinct feature to recognize specific sialylated carbohydrates, usually present at the end of oligosaccharide chains attached to a broad variety of glycoproteins and glycolipids. Glycosylation pattern is heavily altered in BCa, often leading to hypersialylation of the tumor [9]. In this study, we conducted the first analysis of the expression and regulation of Siglec ligands (SL) on bladder tumor cell lines and Siglecs on T cells from BCa patients.

First, the presence of eight SLs was evaluated on non-tumor (URO-tsa and SV-HUC1) and seven tumor urothelial cell lines, by flow cytometry using human recombinant Siglec-Fc (Fig. 1A and Supplementary Fig. 1A and 1B). Of note, since a bimodal shape may be observed (Supplementary Fig. 1A), the expression of S5L and S6L was depicted as percentage (Fig. 1A) or ratio of fluorescence intensity (Supplementary Fig. 1B). Nontumor and tumor urothelial cells do not express S2L and S3L, and a low frequency of cells

**Fig. 1** – SL expression by nontumor and tumor urothelial cell lines and their modulation by BCG infection. (A) Expression (n = 3–4 independent experiments) of S2L, S3L, S5L, S6L, S7L, S8L, S10L, and S14L on nontumor (URO-tsa and SV-HUC1) and tumor (BU68.08, RT4, RT-112, T24, UM-UC3, J82, and TCC-Sup) urothelial cell lines. Values are expressed as the ratio of mean fluorescence intensity for specific staining versus control (RFI), except for Siglec-5 and Siglec-6 ligand labeling, which were displayed as percentage of positive cells. Dotted lines represent the detection threshold. Indicated p values were determined by one-way ANOVA, comparing each tumor cell line with URO-tsa (black stars) or SV-HUC1 (blue stars). (B) Expression of S5L, S6L, and S14L (n = 3 independent experiments) upon treatment with BCG (multiplicity of infection = 100) on indicated cell lines. data are mean ± SEM. ANOVA = analysis of variance; BCG = bacillus Calmette-Guérin; SEM = standard error of the mean; Siglec = sialic acid–binding immunoglobulin domain–containing lectin; SL = Siglec ligand. * p < 0.05, ** p < 0.01, *** p < 0.001.

expressed S6L, S5L, and S14L, which are highly expressed by few bladder tumor cell lines, have comparable patterns of expression (Fig. 1A and Supplementary Fig. 1B). This could be explained by the fact that Siglec-5 and Siglec-14 are paired receptors recognizing similar sialylated ligands [10]. Notably, S7L and S10L were weakly expressed on nontumor cell lines and strongly expressed on tumor urothelial cells (Fig. 1A), suggesting that malignant transformation may trigger cell-surface emergence of these particular SLs. Albeit S9L is highly expressed by tumor cell lines, we observed a discrepancy in its expression by URO-tsa and SV-HUC1. Further investigations are therefore needed to ascertain SL expression on normal and tumor bladder tissue. Next, we studied the influence of BCG infection on SL expression by urothelial cell lines in vitro. Importantly, BCG infection highly increased S5L, S6L, and S14L density on both nontumor and tumor urothelial cells (Fig. 1B and Supplementary Fig. 1B), while other SLs were not affected (Supplementary Fig. 1C).

Next, we characterized the expression of eight SiglecS on T cells of blood from healthy donors (HDs), from NMIBC and muscle-invasive BcA (MIBC) patients, and from tumor tissue of surgical specimens recovered after transurethral resection of bladder tumor or cystectomy (Supplementary Tables 1 and 2). We observed that Siglec-6 is expressed by circulating CD4+ and CD8+ T cells, while other SiglecS were not detectable (Fig. 2A and Supplementary Fig. 2). Interestingly, circulating T cells from NMIBC patients showed higher Siglec-6 expression than those from HDs and MIBC patients. In parallel, tumor-infiltrating lymphocytes (TILs) also express Siglec-6. Yet, no difference in Siglec-6 expression was observed between TILs from NMIBC and MIBC patients (Supplementary Fig. 3).

We then assessed the expression of Siglec-6 on in vitro amplified urinary CD4+ and CD8+ T cells from NMIBC patients after the second and the fifth or sixth BCG instillations (after BCG2 and after BCG5/6). Urinary CD8+ T cells, but not CD4+ T cells, showed increasing expression of Siglec-6 during the course of the BCG therapy (Fig. 2B). Thus, our data show that BCG therapy may not only increase S6L expression on urothelial cells, but also locally increase Siglec-6 expression on bladder-infiltrating CD8+ T cells.

Finally, we determined whether Siglec/SL interaction might inhibit T-cell cytotoxic functions in the context of BcA. Thus, we cocultured purified CD8+ T lymphocytes from HDs or NMIBC patients with BCG-infected tumor urothelial cell lines, which were previously treated or not treated with sialidase. Indeed, sialidase treatment removes sialic acids from cell surface, preventing Siglec/SL interaction and their downstream signaling (data not shown) [5]. We found that
the desialylation of BCG-infected urothelial tumor cells increased the level of CD107a, a surrogate of cytotoxic activity, in CD8+ T cells from HDs and NMIBC patients (Fig. 2C and Supplementary Fig. 4). Altogether, these results indicate that Siglec may limit antitumor immunity elicited by BCG therapy. Since only Siglec-6, among the Siglec we analyzed, is expressed on peripheral T cells, we might speculate that Siglec-6 may be involved in T cell dysfunction.

To investigate whether Siglec-6 expression may influence BCA patient survival, we performed a large-scale analysis from two independent cohorts of transcriptomic datasets from MIBC and NMIBC patients (The Cancer Genome Atlas [11] and UROMOL [12], respectively). High expression of Siglec-6 is significantly associated with poor overall and disease-specific survival in localized and locally advanced BCA (Fig. 2D) and lower progression-free survival in NMIBC (Fig. 2E). These results further suggest that Siglec-6 might be involved in bladder tumor immune escape leading to tumor progression, although we observed a low proportion of bladder tumor cell lines expressing S6L (Fig. 1A and Supplementary Fig. 1B). Additional studies are therefore needed to clarify the expression level of Siglec-6 and S6L in tissue from NMIBC and MIBC patients and their association with the clinical outcome.

Immune checkpoint inhibitors (ICIs) have greatly improved the field of cancer therapy, fostering the research of new immunoregulatory axis. Here, we focused on Siglec, a family of immunoregulatory receptors interacting with sia- loglycans [13]. Similarly to PD-1 and CTLA-4, recent studies highlighted Siglec as potential targets for ICIs, since these can regulate the function of immune cells in the context of inflammation and cancer [7]. Our results suggest that Siglec-6 might be a novel immune checkpoint in BCA, by regulating CD8+ T-cell function, particularly during BCG therapy. However, further validation is warranted to better decide whether the development of Siglec inhibitors might represent a new treatment of particular interest for BCA.

**Author contributions:** Laurent Derré had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study concept and design:** Benmerzoug, Chevalier, Derré.

**Acquisition of data:** Benmerzoug, Chevalier, Schneider, Cesson, Verardo, Villier, Nguyen.

**Analysis and interpretation of data:** Benmerzoug, Derré.

**Drafting of the manuscript:** Benmerzoug, Derré.

**Critical revision of the manuscript for important intellectual content:** Chevalier, Nardelli-Haeßfliger, Jichlinski, Roth, Schneider, Nguyen, Luca, Dartiguenave, Rodrigues-Dias.

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**Administrative, technical, or material support:** Cesson, Dartiguenave, Rodrigues-Dias, Luca, Derré.

**Supervision:** Derré.

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**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at [doi:https://doi.org/10.1016/j.euf.2021.06.001](https://doi.org/10.1016/j.euf.2021.06.001).

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