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Neuropilin-VEGF signaling pathway acts as a key modulator of vascular, lymphatic, and inflammatory cell responses of the bladder to intravesical BCG treatment

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Saban MR, Sferra TJ, Davis CA, Simpson C, Allen A, Maier J, Fowler B, Knowlton N, Birder L, Wu X, Saban R. Neuropilin-VEGF signaling pathway acts as a key modulator of vascular, lymphatic, and inflammatory cell responses of the bladder to intravesical BCG treatment. Am J Physiol Renal Physiol 299:F1245–F1256, 2010. First published September 22, 2010; doi:10.1152/ajprenal.00352.2010.—Recent evidence indicates that VEGF receptors and coreceptors (neuropilins; NRPs) are expressed on nonendothelial cells in human bladder urothelium, in one human bladder cancer cell line (J82), and in the mouse bladder urothelium. In addition, VEGFR1, VEGFR2, NRP1, and NRP2 expressions were upregulated in animal models of chronic bladder inflammation induced by four weekly instillations of protease-activated receptors (PAR)-activating peptides or bacillus Calmette-Guérin (BCG) into the mouse bladder. Here, we used four weekly instillations of BCG as a model for chronic bladder inflammation to further investigate whether VEGF receptors and NRPs play a role in the migration of inflammatory cells and inflammation-induced lymphangiogenesis and angiogenesis. For this purpose, we used neutralizing antibodies that were engineered to specifically block the binding of VEGF to NRP (anti-NRP1B) and the binding of semaphorins to NRP (anti-NRP1A). C57BL/6 mice received intraperitoneal injections of PBS, anti-NRP1A- or anti-NRP1B-neutralizing antibodies and then were challenged chronically with intravesical PBS or BCG. At the end of chronic challenge period, a fluorescent internalizable tracer, scVEGF/Cy5.5, was administered to all mice and near-infrared fluorescence images were obtained in vivo and in real time. BCG increased the overall accumulation of scVEGF/Cy5.5 in the urinary bladder urothelium and inflammatory cells. In addition, BCG increased the density of blood and lymphatic vessels concomitantly with an upregulation of NRP2 expression in lymphatic vessels. Treatment of the mice with NRP1-neutralizing antibodies dramatically reduced scVEGF/Cy5.5 uptake, polymorphonuclear (myeloperoxidase-positive cells) and dendritic cell (CD11c-positive cells) infiltration, and decreased the overall density of BCG-induced blood and lymphatic vessels. These results implicate NRPs as critical in vivo regulators of the vascular and inflammatory responses to the intravesical administration of BCG.

urothelium

Recent evidence places VEGF receptors and its coreceptor (neuropilins; NRPs) in the center of molecular pathways underlying experimental bladder inflammation (50, 57, 63) and represents a key mechanism downstream of inflammation induced by activation of protease-activated receptors (PARs) (63). Indeed, VEGF proteins, receptors, and coreceptors are, in general, increased at the site of inflammation. In addition, infiltrating lymphocytes and other inflammatory cells represent an additional source of VEGF (27, 29). VEGF has been most intensively studied with respect to its actions on vascular endothelial cells, and, in the bladder, increased staining of VEGF was reported in interstitial cystitis patients with glomerulations (pinpoint submucosal hemorrhages) on hydrodistension, but not in patients who failed to show petechial bleeding or in controls (69).

NRPs are transmembrane glycoproteins with a very small cytoplasmic domain and thus rely upon other molecules to transduce their signals across a cell membrane. NRPs were initially identified as coreceptors for plexin with the capacity of binding to and translating the responses of axon guidance factors belonging to the semaphorin family (48). More recently, NRPs were identified as coreceptors for VEGF in arteries, veins, and lymphatic vessels (35) and enhance the binding of VEGF to VEGFR-2 (68). Recent evidence indicates that NRPs and VEGFRs are expressed outside of the vascular system (51). In addition, NRPs are essential for the initiation of the primary immune response (71, 74), are recognized surface markers for regulatory T (Treg) cells (28, 66), and participate in cell-cell communication between antigen-presenting (14) and effector cells (12, 15). Of interest for the present work, NRPs are strongly expressed in normal human bladder biopsies (64) in association with VEGF receptors (55) and in the human urothelium carcinoma cell line (J82) (64). In addition to the urothelium, NRPs are also expressed in the mouse bladder vascular system, detrusor smooth muscle, and intramural ganglia (55). Other investigators have shown by in situ hybridization an intense expression of NRP in the mouse bladder detrusor muscle at embryonic day 15.5 (E15.5) (18), and in human bladder cancer NRP2 was among the top-ranked molecular target differentially expressed and validated by immune histochemistry (55). As VEGF receptors and NRPs seem to play an important role in inflammation and immunity, we sought to confirm that the VEGF pathway is part of the basic mechanism underlying chronic bladder inflammation. Compu-
tional models simulating in vivo VEGF transport and binding to its receptors (40) and in vivo animal models (39) indicate that blockade of NRP-VEGFR coupling is significantly more effective than any other approach in decreasing VEGF signaling. As monoclonal antibodies were engineered to alter NRP-VEGFR coupling and represent one of the best therapies to block VEGFR-mediated events (49), we used anti-NRP1A that was developed to neutralize the site of NRP1 responsible for semaphorin binding (a1-a2) and anti-NRP1B that neutralizes the NRPI site responsible for VEGF binding (b1-b2) to test the hypothesis that VEGF signaling plays an important role in bladder inflammation.

The innovative aspect of this manuscript was to test the use of potent and specific NRP antibodies to reduce inflammation, edema, and vascular permeability in an established mouse model of chronic bladder inflammation. In addition, this manuscript describes the application of molecular imaging as a resource for real-time and in vivo assessment of VEGF pathway antagonism.

METHODS

Experimental bladder inflammation. All animal experimentation described here was performed in conformity with the APS “Guiding Principles in the Care and Use of Animals” and University of Oklahoma Health Sciences Center Animal Care and Use Committee protocol 08–105. In this study, mice received four weekly instillations (i.v.) of PBS (150 μl) on day 0, and then twice a week for 5 wk, by intravesical instillation of 150 μl of PBS or BCG (TheraCys; total dose 1.35 mg, Sanofi-Pasteur) (Ref. 61, Fig 1). For this purpose, mice were anesthetized with ketamine HCl (40 mg/kg im) and xylazine (2.5 mg/kg im), and a polypropylene catheter (24 gauge; ¾ in.; Angiocath, Becton-Dickinson, Sandy, UT) was introduced transurethrally into the bladder and advanced until the first drop of urine appeared. After drainage of urine, by application of light pressure to the abdomen, one of the above substances was introduced into the bladder lumen, at a slow rate to avoid trauma and vesicoureteral reflux. To ensure consistent contact of substances with the bladder and to avoid reflux or leakage, the catheter was closed with a stopcock (VWR) for 30 min.

On day 37, mice were injected intravenously, via the tail vein, with 100 μl of a tracer, scVEGF/Cy5.5, containing ~0.5 nmol of the tracer, and near-infrared fluorescence (NIRF) images were acquired between days 37 and 38. On day 38, mice were euthanized and tissues were removed for histology and immunofluorescence (IF). NIRF imaging. Before imaging, mice were fed a low-chlorophyll diet for 2 wk to reduce autofluorescence in the intestinal region (72), and the hair was removed. Mice were anesthetized with isoflurane and intravenously injected, via the tail vein, with 100 μl of ~0.5 nmol of scVEGF/Cy5.5. Anesthetized mice were immediately placed in a supine position on a heating pad inside a FluorChem HD2 cabinet (Alpha Innotech, San Leandro, CA) equipped with a Chromalight multiwavelength illuminator with a Cy5 excitation filter (620 ± 60
nm), and images were captured with 4-megapixel cooled camera (F2.8, 28- to 70-mm zoom lens) with a Qdor 705 emission filter (705 ± 10 nm). Images were first acquired and stored with AlphaEase FC 32-bit software (Alpha Innotech). Following NIRF, mice were euthanized with pentobarbital sodium (100 mg/kg ip) and tissues were removed rapidly and frozen for IF.

NIRF image analysis. NIRF image analysis was performed, as we described previously (62). Briefly, images were first acquired and stored with AlphaEase FC 32-bit software (Alpha Innotech), and next the use of Adobe Photoshop CS4 Extended permitted the determination of integrated density, as described (62). At each time point subsequent to scVEGF/Cy5.5 intravenous administration, the integrated fluorescence intensity was determined as the equivalent of the product of the area (in pixels) and mean gray value. Background levels of fluorescence (time 0) were subtracted for each time point. The time course data were analyzed through a 2-way ANOVA. There was a significant time-by-treatment interaction (P < 0.0001), so the data were stratified by time and analyzed in four one-way ANOVAs. An α of 0.05 was considered statistically significant.

RESULTS

Biweekly treatment with NRPIA or NRPIB antibodies for 4 wk did not induce any noticeable change in animal behavior, food consumption, and body weight (data not shown). To identify cells with accessible and functionally active VEGF receptors, we tagged such cells in vivo with a new, internalizable fluorescent tracer, scVEGF/Cy5.5 (7, 55) that is capable of detecting, in vivo and in real time, alterations in VEGF receptor activity induced by inflammation (55, 64). Importantly, unlike immunohistochemical analysis that shows all cells expressing VEGF receptors, receptor-mediated tagging with the scVEGF/Cy5.5 tracer identifies only cells with accessible and functionally active VEGF receptors (4, 6, 7). We previously presented evidence that inVEGF/Cy5.5, a control fluorescent tracer based on scVEGF/Cy5.5 and incapable of binding to VEGF receptors, does not lead to the appearance of fluorescence in the lower abdominal area of mice or in cross sections of removed tissues (55).

We compared the time course of scVEGF/Cy5.5 uptake in mice instilled with PBS (Fig. 2A) vs. those that received chronic BCG (Fig. 2B). We found similar kinetics and levels of tracer uptake in PBS- and BCG-treated mice up to ~120 min postinjection (Fig. 2E). In control animals, after a peak at 2 h postinjection, the observed scVEGF/Cy5.5 fluorescence rapidly declined, whereas in BCG-treated mice, higher levels of fluorescence were observed for far longer periods of time (Fig. 2E). In contrast, mice that received anti-NRP antibodies concomitantly with chronic BCG instillation had a reduced accumulation of scVEGF/Cy5.5 (Fig. 2, C and D) that was reflected by a decrease in the integrated fluorescence density back to control levels (Fig. 2E). Together, these results suggest that the observed effects of intravesical BCG on VEGF accumulation were strongly reduced by systemically administered NRPI neutralizing antibody.
Ex vivo localization of scVEGF-Cy5.5. We previously showed that systemic administration of scVEGF-Cy5.5 resulted in strong accumulation of the tracer in the bladder epithelium in cells expressing high levels of VEGFRs and NRPs and that inflammation induces both upregulation of these receptors and increased scVEGF-Cy5.5 accumulation (55). In the present study, we confirmed that scVEGF-Cy5.5 accumulates in the urothelium (not shown). Moreover, a strong accumulation of scVEGF-Cy5.5 was observed in lamina propria (Fig. 3A; red arrows), and an intense accumulation of scVEGF-Cy5.5 was observed in CD11c-positive dendritic cells (Fig. 3, B–D) and F4/80-positive macrophages migrating into the urinary bladder in response to BCG (Fig. 3, E–G). As scVEGF-Cy5.5 labels only cells expressing active VEGF-NRP receptors (7), these results further suggest a role for these receptors in the immune response (45, 66).

Anti-NRP1 antibodies reduce BCG-induced accumulation of inflammatory cells. The strong accumulation of scVEGF-Cy5.5 in CD11c- and F4/80-positive cells suggests that the VEGF pathway may also be involved in the infiltration of these inflammatory cells into the urinary bladder. To test this hypothesis, we determined whether concomitant treatment of BCG- and anti-NRP1-neutralizing antibodies would result in a reduction of inflammatory cells compared with the group receiving PBS-BCG. Figure 3, H–J, show representative tissue sections for CD11c- and F4/80-positive cells obtained in bladders isolated from PBS-BCG-treated mice. Few of these cells were double positive for CD11c and F4/80 and were excluded from the quantification (Fig. 3J; white arrows). Image analysis indicates a predominance of F4/80-positive cells over CD11c-positive cells per cross section that were mobilized by intravesical BCG treatment (Fig. 4, A and B). Neither of the antibodies reduced the number of F4/80-positive cells. Interestingly, treatment of mice with anti-NRP1A-neutralizing antibody significantly reduced the number of CD11c-positive cells migrating in response to BCG, whereas NRP1B had no significant effect. The finding that F4/80 cells were not altered suggests that the effect of the NRP1A antibody on CD11c-
positive cells was not due to an overall reduction of the chronic inflammation. However, treatment of mice with anti-NRP antibodies prevented the increase in myeloperoxidase-positive PMNs infiltration that is characteristic of the bladder response to BCG (58, 65) (Fig. 4C). These results add to previous findings indicating an important role for VEGFRs and NRPs in experimental bladder inflammation (55, 63).

BCG-induced lymphangiogenesis. Recently, several markers of lymphatic endothelial cells were introduced, including a receptor for hyaluronan (LYVE-1) (10), VEGFR-3, Flt-4 (fms-like tyrosine kinase 4; Flt-4), and podoplanin (D2–40 antibody) (26). However, LYVE-1 is also expressed in some blood vessels and macrophages, VEGFR-3 is also expressed on some blood vessels, and podoplanin is also expressed on some epithelial cells and podocytes (see Ref. 8 for a review). In addition, the hyaluronan receptor that is labeled with LYVE-1 antibody is downregulated by proinflammatory cytokines (33), making the use of this stain alone difficult in determining the effects of inflammation-induced lymphangiogenesis. In the present work, we found that lymphatic vessels are strongly concentrated in the lamina propria of the urinary bladder just below the urothelial cell layer (Fig. 5, A–C). In addition, lymphatic vessels were found in the detrusor between muscle bundles (Fig. 5, D–F), and large collecting lymphatics were

Fig. 3. scVEGF-Cy5.5 accumulation in the bladder urothelium, blood vessels, and migrating inflammatory cells. Shown are representative photomicrographs from the urinary bladder isolated from group II (PBS/BCG) 24 h after systemic administration of scVEGF-Cy5.5. Cy5.5 fluorescence signals were converted with NIS-Elements software to white to contrast with other immunostains. 4',6-Diamidino-2-phenylindole (DAPI) highlights the cell nuclei. Note the strong accumulation of the tracer in blood vessels of the lamina propria (A; red arrows), CD11c-positive cells (B–D, white arrows), and F4/80-positive cells (E–G). For clarity, another set of pictures illustrate that some of CD11c- (3H) and F4/80-positive cells (I) were double positively stained (J, white arrows).
observed in the bladder adventitia, as we described previously (59). However, lymphatic markers such as LYVE-1 and podoplanin showed different staining patterns depending on their specific location (Fig. 5; green arrows indicate podoplanin-positive, LYVE-1-negative cells). In addition, podoplanin was expressed not only in lymphatic vessels but also in stromal cells in the lamina propria, just below the urothelial cell layer forming a fine meshwork surrounding the lymphatic vessels and arteries (not shown). Similar results with podoplanin were found in the mouse spleen, and it was suggested that the podoplanin-positive meshwork acts as an extravascular lymphatic pathway and, that together with ordinary lymphatic vessels, plays a primary role in the cell traffic of the spleen, additional to the blood circulatory system (67). The precise function of this podoplanin-positive meshwork in the urinary bladder remains to be determined.

Because of our findings regarding the heterogeneity of staining of lymphatic markers, we took the risk of underestimating the number of lymphatic vessels and considered only LYVE-1 and podoplanin double-positive vessels (Fig. 5; yellow arrows) as representative of lymphatic vessel density. To determine whether BCG induces proliferation of preexisting lymphatics, we applied the REMARK guidelines (75) that define lymphangiogenesis by the colocalization of specific markers of lymphatic vessels and those for cell proliferation (Ki-67), a nuclear protein that is associated with and may be...
necessary for cellular proliferation (20, 75). The results presented in Fig. 6, A–C, indicate that BCG induces bladder lymphangiogenesis similar to that observed in bladder cancer (26, 44, 62). As a consequence, BCG induces a significant increase in lymphatic vessel density (Fig. 6D).

**Anti-NRP1 antibodies prevent the increase in lymphatic vessel density.** Pretreatment of animals with either anti-NRP1\(^A\) or anti-NRP1\(^B\) prevented the BCG-induced increased lymphatic vessel density (Fig. 6D) and lymphangiogenesis, as characterized by the absence of coexpression of Ki-67 and podoplanin (not shown). These results implicate the participation of NRPs as a mechanism by which BCG leads to activation of lymphatic vessel plasticity. We previously described the capacity of BCG to upregulate the message and expression of NRP1, NRP2, VEGFRs, and VEGF-C (55). As NRP2 is known to play a role in lymphangiogenesis (2, 70), we sought to investigate whether the BCG-induced increased NRP2 expression extended from the urothelium (55) to the bladder lymphatic vasculature. As previously described, NRP2 is not only highly expressed in the bladder urothelium (Fig. 7C; white wave-like areas), but also in podoplanin-positive lymphatic vessels (Fig. 7; white arrows). Quantification of NRP2 expression was performed taking the area occupied by podoplanin-positive vessels as the ROI, and the image analysis results indicate that BCG treatment resulted in increased expression of NRP2 in lymphatic vessels (Fig. 7G). Therefore, one possible mechanism underlying BCG-induced lymphangiogenesis seems to be the upregulation of NRP2 expression in lymphatic vessels. Finally, NRP-neutralizing antibodies prevented the BCG-induced increase in lymphatic vessel density, further indicating a key role for NRPs in bladder lymphangiogenesis.

**Anti-NRP1 antibodies prevent the increase in blood vessel density.** BCG induced a strong vascular response of the urinary bladder, with dilation of suburothelial blood vessels, as illustrated in Fig. 8, A and B, and quantified by the image analysis of the area occupied by CD31-positive cells (Fig. 8C). Although in mice treated with NRP1\(^A\) there was a trend for a reduction in the response of blood vessels to chronic BCG, the values did not reach statistical significance. However, NRP1\(^B\)

![Fig. 6. BCG-induced bladder lymphangiogenesis. Shown are representative photomicrographs from the urinary bladder isolated from group II (PBS/BCG) indicating that some of the podoplanin-positive lymphatic vessels (A) also labeled positively for Ki-67, a marker of cell proliferation (B). Merged images (C) illustrate lymphangiogenesis, as defined by the REMARK guidelines (75). D: illustrates the finding that NRP antibodies prevent BCG-induced increase in lymphatic vessel density. Lymphatic vessel density of bladder cross sections isolated from groups I–IV was defined as means ± SE of LYVE-1 and podoplanin (D2–40) double positive as a percentage of the total bladder cross-sectional area (n = 12/group). Chronic BCG instillation induced a statistically significant increase in the area covered by lymphatic vessels, whereas pretreatment with NRP1\(^A\) or NRP1\(^B\) prevented the effects of BCG on lymphatic vessel density. Horizontal bars indicate a statistically significant difference with its respective P value.](http://ajprenal.physiology.org/)}
prevented the increase in both blood vessel density (Fig. 8) and lymphatic vessel density (Fig. 6).

DISCUSSION

The purpose of this study was to investigate the role of the VEGF pathway, and in particular the NRPs, as a major contributor for BCG-induced plasticity of blood, lymphatic vessels, and inflammatory/immune cells. We chose to use four weekly instillations of BCG to investigate the role of VEGF because it is known that this regimen induces increased expression of VEGF and upregulation of VEGFR1, VEGFR2, NRP1, and NRP2 in the mouse bladder. In contrast, the continuation of intravesical instillations for 6 wk, as it is suggested as a treatment for humans with bladder cancer, leads to the production of interferons and other inhibitory factors that reduce the angiogenic response (13, 50), and therefore would have made it impossible to test the present hypothesis.

Mechanistically, it has to be taken into consideration that NRPs are glycoproteins that present just a reminiscence of intracellular domain. As such, NRPs themselves do not induce a signal transduction pathway. It is the coreceptor function of NRPs that was investigated. As coreceptors for VEGFRs and plexin, the signal transduction pathways being studied are those elicited by VEGF or semaphorin. Our results clearly showed that both anti-NRP antibodies reduced the uptake of the fluorescent tracer scVEGF/Cy5.5 in the urinary bladder of mice receiving chronic BCG concomitantly with a reduction of inflammation and vascular remodeling. Our results obtained with the NRP1B antibody that neutralizes the NRP1 domain responsible for VEGF binding suggest the involvement of this growth factor on bladder responses to BCG. In contrast, the results obtained with the NRP1A antibody suggest the involvement of semaphorins on inflammatory cell migration and lymphangiogenesis. Supporting this hypothesis, our preliminary PCR results indicate that both the bladder mucosa as well as the detrusor muscle express the message for plexin A2 and, to a lesser extent, plexin A1 that function as NRP coreceptors (data not shown). Therefore, future studies should be focused on elucidation of a role for semaphorins on bladder immune responses to BCG therapy.

In addition, our results demonstrate strong evidence of the association between BCG administration and inflammatory
cells that express active VEGF-NRP receptors, as indicated by the accumulation of scVEGF/Cy5.5 in CD11c- and F4/80-positive cells. As scVEGF/Cy5.5 is an internalizable tracer which accumulates specifically in cells expressing active VEGF receptors (4, 5), and it is retained within these cells even when its circulating levels approach zero (7), our results support previous findings that active VEGF/NRP receptors are present in migrating inflammatory cells (14, 15, 28, 45, 66). Although the activity of these migrating cells was not investigated, it seems reasonable to propose that future studies should focus on the effect of NRP antibodies on CD11c(+)-dependent cytokine production, such as IL-12/23p40 (54) and IL-17 that are integral parts of BCG therapy (61). It is known that NRP1 is expressed by most regulatory T cells (66), but there is little information available on the expression of NRP2 in human immune cells, with the exception of dendritic cells (21). More recently, it was shown that the expression of Sema3A receptors (NRP1, NRP2, plexin A1, plexin A2, and plexin A3) was significantly increased during M-CSF-mediated differentiation of monocytes into macrophages (32). One of the limitations inherent in in vivo study is the difficulty of extracting inflammatory cells from the bladder and sorting them by flow cytometry, which is the gold standard approach for the study of immune cells. Therefore, our conclusions regarding the specific immune cell subtype should be taken in light of the limitations of IF. Nevertheless, our results strongly suggest that BCG increased the tissue migration of inflammatory cells by a mechanism that is reduced, at least in part, by NRP1. It remains to be determined whether other antibodies specifically targeting NRP2 (17) would present better efficacy in reducing CD11c- and F4/80-positive cells that migrate in response to BCG.

In addition to its effects on the migration of inflammatory cells, we found that BCG induces a strong vascular response that is translated by increased density of blood and lymphatic vessels. As lymphatic vessels regulate tissue fluid homeostasis and immune cell trafficking, the initial increase in lymphatic vessel density is proposed as a basic mechanism underlining the beneficial effects of intravesical BCG by improving drainage of the extracellular fluid, reducing tissue edema, and improving the migration of immune cells. Others have shown the participation of lymphatics in the immune response to Mycobacterium tuberculosis (Mtb) by infection with BCG-GFP and detection of bacteria within the lymphatics in response to intranasal (11, 31), oral (23), or intradermal immunizations (1). NRPs and dendritic cells carrying fluorescent bacilli leave the inflamed site via afferent lymphatics, migrate to the secondary lymphoid organ, and participate in the transport of live microorganisms (1). In addition to BCG, other bacterial infections such as Mycoplasma pulmonis resulted in robust lymphatic and blood vessel remodeling that depended on an intact immune system (3). Moreover, M. pulmonis induces lymphangiogenesis that is driven by VEGF-C- and VEGF-D-expressing immune cells and could be inhibited by using a VEGF-C/D trap20 (9). Our results strongly indicate that VEGF/NRP receptors are involved in the bladder vascular response to BCG. Interestingly, we are also reporting for the first time that NRP2 is strongly expressed in bladder lymphatic vessels and that BCG treatment upregulates its expression.
Finally, both NRP antibodies reduce the plasticity of blood and lymphatic vessels induced by BCG.

The finding that BCG can induce lymphangiogenesis and that anti-NRP antibodies reduce such a response provides new tools for the study of basic mechanisms involved in the regulation of bladder lymphatic physiology and pathophysiology. These studies can lead to a better understanding of the role of lymphatic vessel plasticity in response to urinary tract infections, inflammation, and cancer development (62). A fundamental role for immune cells, macrophages in particular, has been postulated in inflammation-induced-lymphangiogenesis. Supporting this hypothesis, evidence has been presented indicating a major role for macrophages in VEGF-3 ligand-induced angiogenesis (19) and in VEGFR-1 signaling promoting lymphangiogenesis as well as angiogenesis (46). In addition, a mandatory role for CD11b-positive macrophages has been proposed in inflammation-induced-lymphangiogenesis of the cornea (42). Additional support for a role for immune cells in vessel plasticity is the finding that when macrophage activity is reduced, as is the case in diabetic mice, LYVE-1-positive lymphatic vessels and CD31-positive blood vessels are found to be significantly reduced in corneal wound healing in diabetic mice (41). Moreover, interleukin-1β stimulation rescued diabetic macrophage function and induced lymphatic vessel formation (41). However, macrophages are not the only immune cells involved in blood and lymphatic vessel plasticity, and recent reports indicate that in addition to macrophages, mast cells play a fundamental role in development of tumor-associated blood and lymphatic capillaries (73). The strong correlation between immune cell migration and the plasticity of blood and lymphatic vessels justifies the concomitant presentation of our results regarding the effect of anti-NRP antibodies on BCG-induced immune cell migration and vascular plasticity.

It is premature, to say the least, to use the present results to define the exact mechanisms underlying bladder inflammation. However, a working hypothesis is that regardless of the cause of urothelial injury, i.e., BCG, bacterial infection, or any noxious substance in the urine, the urinary bladder responds by increasing the production of VEGF that acts initially as a survival factor (16) but also has the capacity of increasing vascular permeability (24), leading to glomerulations (34, 69), edema, and inflammation. The present findings add to existing evidence indicating that, in the urinary bladder, NRPs are expressed in the urothelium, immune cells, nerves, blood vessels, and lymphatic vessels and mediate the accumulation of VEGF tracer. Our results clearly showed that systemic treatment of the mice with NRP1-neutralizing antibodies dramatically blunted the chronic inflammation induced by BCG, as indicated by reduction of scVEGF/Cy5.5 uptake, immune cell infiltration, and the overall density of blood and lymphatic vessels. Therefore, it is fair to conclude that NRPs, VEGF, and semaphorins play a role in several steps of the bladder responses to proinflammatory stimuli. The above results illustrate the tremendous difficulty in defining where and how the chronic inflammatory responses to intravesical BCG are initiated and propagated. To answer this question, cell-specific deletion of NRPs should be performed since knockout mice present fundamental problems of development. The advent of floxNRP1 and floxNRP2 mice permit cell-specific deletion of these receptors, and experiments in our laboratory are under-way addressing the role of urothelial NRPs on the cascade of events subsequent to BCG administration.

We conclude that intravesical BCG therapy increases blood vessel density and induces proliferation of preexisting lymphatics, concomitantly with an increase in expression of NRPs and VEGFRs (55). In contrast, interstitial cystitis patients presented a significant reduction of VEGFR1 and NRP2 expression in the bladder urothelium and, therefore, those patients may benefit from intravesical BCG therapy. In addition, the use of anti-NRP antibodies concomitantly with BCG therapy confirmed previous observations that the bladder immune and vascular responses to BCG are mediated by the VEGF/NRP pathway (58, 60). Both NRP1A and NRP1B antibodies fundamentally altered the immune and vascular response to chronic BCG treatment and reduced the inflammatory arm of BCG therapy. Further experiments on the concomitant use of BCG and NRP antibodies are necessary to determine whether the beneficial effects of BCG in interstitial cystitis and bladder cancer remain despite a reduction of the proinflammatory arm of this therapy.

Perspectives and significance. In summary, the present work adds VEGFRs and NRPs as an additional layer of the complex bladder responses to immune-stimulating agents. A better understanding of how the expression of these guidance molecules control both the immune and vascular system will lead to a better understanding of the pathophysiology of the lower urinary tract.

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DISCLOSURES

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NEUROPILIN-VEGF PATHWAY AND BCG


