Neutrophil Granulocytes Are Required for Effective *Bacillus Calmette-Guérin* Immunotherapy of Bladder Cancer and Orchestrate Local Immune Responses

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Abstract

The role of polymorphonuclear neutrophil granulocytes (PMN) in antitumoral immune responses displays a striking dichotomy. Under inflammatory conditions, PMN may promote tumor growth and progression. In contrast, especially in the context of therapeutic interventions, PMN can exert important antitumor functions. However, until now, the mechanisms of PMN-mediated activation of tumor immunity are poorly defined. Based on a murine model of *Bacillus Calmette-Guérin* (*BCG*) immunotherapy of bladder cancer, we provide evidence for a novel immunoregulatory role of this leukocyte subset. PMN immigrate into the bladder after intravesical BCG instillation and depletion of PMN from tumor-bearing mice completely abrogated antitumor efficacy of BCG. PMN stimulated with BCG *in vitro* as well as PMN isolated from the urine of BCG-treated patients were a major source of the chemokines interleukin-8, growth-related oncogene-α, macrophage inflammatory protein-1α and of the inflammatory cytokine migration inhibitory factor. *In vitro*, BCG-stimulated PMN indirectly induced T-cell chemotaxis via the accessory function of activated monocytes. *In vivo*, depletion of PMN from BCG-treated mice significantly impaired $CD4^+$ T-cell trafficking to the bladder. These data show that PMN direct the migration of effector cells to the bladder and by this means are indispensable for effective tumor immunotherapy. Thus, our findings provide evidence for a novel early immunoregulatory role of these innate immune cells in local antitumor immunity. (Cancer Res 2006; 66(16): 8250-7)

Introduction

Bladder cancer is the fifth most common malignant disease, with an incidence that reached >63,000 new cases in the United States alone in 2005 (1). On initial diagnosis, 75% to 80% of cancers are nonmuscle invasive tumors of stages pTa, pT1, or pTis. Depending on grade and stage, these tumors display highly variable rates of recurrence and progression at 5 years following transurethral resection (TUR) of up to 78% and 45%, respectively (2). In 1976, Morales et al. (3) reported for the first time on the successful intravesical use of *Mycobacterium bovis Bacillus Calmette-Guérin* (*BCG*) as an adjuvant treatment option following TUR. BCG immunotherapy consists of 6 weekly intravesical instillations. In high-risk tumors, an additional maintenance schedule is recommended (4). The mycobacteria induce a strong local immune response within the urinary bladder, characterized by the massive secretion of cytokines into the urine as well as inflammatory cellular infiltration (5). Today, BCG immunotherapy has been shown to be the only efficient adjuvant treatment option for preventing or delaying progression after TUR of nonmuscle invasive bladder cancers (6, 7) and represents one of the most successful immunotherapies of solid cancer.

Since the first use, many aspects of the complex immune response to BCG have been revealed. Following intravesical instillation, BCG provokes an early influx of innate immune cells, such as polymorphonuclear neutrophil granulocytes (PMN) and a proinflammatory cytokine milieu of predominantly T_H1 type (5, 8, 9). After repeated instillations, the bladder wall is infiltrated by mononuclear cells, consisting mainly of monocytes/macrophages, $CD4^+$ and $CD8^+$ T lymphocytes, as well as natural killer (NK) cells, which form chronic granuloma-like cellular infiltrates in the suburothelial stroma (10). In addition to descriptive studies in humans, an orthotopic murine bladder cancer model was established to do functional investigations (11). Using this model, it was shown that T_H1-polarized cell-mediated immunity plays a key role during BCG immunotherapy *in vivo* and that IFN-γ, NK cells, and $CD8^+$ as well as $CD4^+$ T cells are major cellular mediators of this antitumor action (12–14).

Although T-cell–mediated cell lysis and the release of regulatory cytokines, such as IFN-α and IFN-γ, have been shown to represent late acquired immune events of the antitumor effector phase, thus far little attention has been paid to the early innate immune response that immediately follows BCG instillation (5). PMN are such early innate immune cells and the predominant subpopulation of leukocytes in the urine after BCG instillation (8, 9). Having long been regarded simply as phagocytic effector cells and potential mediators of BCG side effects, some studies have of late challenged this opinion. Today, PMN have been shown to be of critical importance in the early inflammatory response against mycobacterial infections, probably by providing an important link between innate and acquired immune responses (15, 16). Furthermore, their role in malignant tumor growth and tumor immunology is currently a matter of intensive debate. Although most studies suggest a tumor-promoting "nursing" function of PMN in advanced disease (17), under certain circumstances, they also seem to exert tumor-inhibitory actions during early cancer cell growth (18, 19).

Previously, we have been able to show extensive alterations in gene expression, enhanced interleukin (IL)-8 secretion and

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prolongation of survival in PMN following BCG stimulation (20). These data are in concordance with recent evidence that activated PMN act as an important source of chemokines of the CXC [e.g., IL-8 and growth-related oncogene (GRO)-α], as well as CC (e.g., macrophage inflammatory protein (MIP)-1α and MIP-1β), subfamily in response to mycobacterial stimulation *in vitro* (21). Yet, the implications of these observations *in vivo* are unknown. Functional studies on immune cell activation, chemotaxis, and trafficking in response to *Mycobacterium*-stimulated PMN are lacking. Clinical observations support a potential functional role for PMN during BCG immunotherapy. Local concentrations of IL-8 in the bladder were shown to predict clinical responses to BCG (22). The extent of leukocyturia as well as local irritative symptoms and fever correlate with a favorable outcome after BCG administration (23, 24). Stimulated by these observations, we hypothesized that via secretion of chemotactic factors, PMN might exert essential immunoregulatory functions during BCG immunotherapy by directing leukocyte traffic in the early inflammatory response in the bladder of patients. In fact, we show here for the first time that PMN isolated from the urine of BCG-treated patients are a relevant source of CC as well as CXC chemokines. *In vitro* and in a well-defined orthotopic murine bladder tumor model, activated PMN attract monocytes, which in turn results in BCG-induced $CD4^+$ T-cell migration. Our findings define PMN as indispensable immunoregulatory cells during BCG immunotherapy.

Materials and Methods

Reagents and cell lines. Viable mycobacteria (BCG, Connaught substrain; kindly provided by Cytochemia, Ihringen, Germany) from the logarithmic growth phase were used for stimulation experiments. The number of colony-forming units (CFU) was routinely determined by plating and incubation at 37°C for 4 weeks on Löwenstein-Jensen agar. The murine bladder tumor cell line MB49 [kind gift of E.C. de Boer (University of Amsterdam, Amsterdam, the Netherlands) and M.A. O'Donnell (University of Iowa, Iowa City, IA)] was maintained in RPMI 1640 culture medium (Biochrom, Berlin, Germany) supplemented with 10% low-endotoxin FCS, penicillin, and streptomycin, hereafter referred to as complete medium, at 37°C in 5% CO_2. The rat hybridoma cell line RB6-8C5 producing the anti-Gr-1 antibody was a kind gift from T. Laskay (Institute for Medical Microbiology and Hygiene, Lübeck University, Lübeck, Germany) and maintained in complete medium containing 1% FCS. For antibody preparation, supernatants were purified on sepharose G columns.

Murine bladder cancer model and PMN depletion. We used a well-defined murine syngeneic orthotopic MB49 bladder cancer model to evaluate the role of PMN for effective BCG immunotherapy *in vivo* (11). In brief, 6- to 8-week-old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were catheterized to receive an intravesical inoculate of 10^5 MB49 bladder tumor cells on day 0 following 5 seconds of electrocautery of the bladder wall. On days 1, 8, 15, and 22 following tumor implantation, mice were treated intravesically with either 3×10^6 CFU BCG or PBS control for a total of four instillations. Groups of eight mice each were followed daily for survival or terminal morbidity. To do immunohistochemical bladder wall stainings, groups of six mice each were sacrificed on day 9 and the bladders were processed for cryosections. PMN were depleted from BCG- or PBS-treated mice using an anti-Gr-1 monoclonal antibody (mAb) or a respective isotype control according to a modified protocol as described (25). RB6-8C5 (250 μg) or the isotype control antibody (250 μg) was given i.p. every 2 to 3 days as indicated in the respective figure legends. Efficacy of depletion was constantly monitored during experiments by counting the relative numbers of PMN on Giemsa-stained whole mouse blood smears. RB6-8C5 treatment reduced PMN blood counts to <10% of pretreatment levels. Neutropenia persisted for 10 days after which repletion to levels equal to or even greater than pretreatment levels occurred. In control experiments, 100% of tumor-free mice survived the antibody injections (data not shown). All animal experiments done during this study were approved by the ministry for environmental, agricultural, and forestry affairs of the county of Schleswig-Holstein, Germany.

Immunohistochemistry. Mouse bladders were resected, shock frozen in liquid nitrogen, and stored at −80°C. Immunohistochemical detection of various immune cell subpopulations was done on 5-μm cryostat sections following a routine staining protocol using peroxidase-conjugated secondary antibodies as published previously (12, 13). Antibodies and their respective isotype controls against the listed mouse antigens were as follows: CD4 (clone RM4-5, 500 ng/mL; BD Biosciences, Heidelberg, Germany), Gr-1 (clone RB6-8C5, 700 ng/mL; see above), and CD11c (clone N418, 1 μg/mL; Serotec, Düsseldorf, Germany). To quantify the cellular bladder infiltrate, groups of six mice were used. The number of infiltrating immune cells was determined by examining 7 to 12 randomly selected nonoverlapping microscopic fields (0.126 mm^2) at ×250 magnification. Counting of cells was done in blinded fashion by two independent individuals.

Patients, healthy donors, and urine and peripheral blood samples. Ten urine samples from BCG-treated patients were collected 20 to 24 hours past the first, second, or third instillation of the induction cycle. Simultaneously, peripheral venous blood was drawn from the respective patients as well as from healthy volunteers. Urinalysis of pre-BCG urine samples from all individuals revealed clean urine devoid of cellular or bacterial components. Urine samples were immediately processed by centrifugation and washed twice in complete medium. These cell suspensions contained >95% PMN as confirmed by Giemsa stainings and flow cytometry. Cell suspensions of peripheral blood PMN from patients and healthy volunteers were obtained according to a standard protocol described recently (20). The experiments were approved by the ethics committee of the Lübeck University Medical School (#02-137) after obtaining written informed consent from all individuals.

PMN purification protocol. To remove contaminating monocytes and lymphocytes from PMN in patients' urine and peripheral blood before doing ELISAs, we developed an antibody-based purification protocol. Cell suspensions containing peripheral blood PMN and urine PMN were labeled at a concentration of 3×10^7/mL with 1 μg/mL mAb directed against CD66b (clone 80H3; BD/PharMingen, Heidelberg, Germany) and subsequently coated with goat anti-mouse immunomagnetic microbeads (Miltenyi; Bergisch Gladbach, Heidelberg, Germany). CD66b is constitutively expressed on PMN, induced on activation, and has been identified as a major galectin-3 receptor (26). Cells were processed on LS VarioMACS separation columns (Miltenyi) and washed thrice before eluting them in MACS buffer containing 2 mmol/L EDTA and 0.5% bovine serum albumin. Purity of PMN in the eluate was >99.9% as determined by extensive flow cytometric analyses. Using FITC-conjugated anti-CD66b mAbs, we did not detect any differences in the expression of CD66b on peripheral blood PMN and urine PMN before and after separation. These data are in concordance with a recent publication, which showed effective PMN isolation with anti-CD15 immunobeads without cell activation (27).

Human IL-8, GRO-α, MIP-1α, and migration inhibitory factor ELISA. Following purification, peripheral blood PMN and urine PMN were analyzed for chemokine secretion using commercially available assay kits supplied by R&D systems (Minneapolis, MI) according to the manufacturer's instructions.

Monocyte and T-cell chemotaxis assay. A method to assay monocyte and T-cell chemotaxis was developed using a modification of the endogenous component chemotaxis assay described previously (28). Monocytes were isolated from peripheral blood mononuclear cells (PBMC) from healthy human donors by counterflow centrifugation (elutriation). This method yields monocyte suspensions with >99% purity. T cells were generated by culture of PBMC in complete medium supplemented with 2.5 μg/mL phytohemagglutinin for 3 days and subsequently for 6 days in complete medium supplemented with 200 units/mL recombinant human IL-2. After this procedure, cultures contained T cells of >98% purity as determined by flow cytometry. Monocytes (10^7) or T cells (10^7) were radioactively labeled in complete medium with 200 μCi $Na_2^{51}CrO_4$ for

2 hours. Labeled cells (0.5×10^6) in 200 μL complete medium were placed into the upper compartments of a 24-Transwell Costar plate (Corning, New York, NY) with 5-μm pore membranes. The lower compartments contained the various chemoattractants in 500 μL complete medium as indicated in figure legends. At different time points, cells in the lower compartments and cells detached from the lower side of the pore membranes were lysed in 0.1% Triton X-100 and radioactive release in supernatants was determined in a gamma counter (Berthold LB2111, Bad Wildbach, Germany). Percentage of migrated cells was determined in relation to the maximal radioactive release of 0.5×10^6 lysed labeled cells (representing 100% migration).

Statistical analyses. Survival of mice was evaluated using Kaplan-Meier plots and the log-rank test. Significance of differences in leukocyte bladder wall infiltration and PMN chemokine secretion was determined using a Mann-Whitney *U* test. Results are shown as mean with error bars representing ± SD. Statistical analyses are given as two-sided *P*s with a $P < 0.05$ being considered a statistically significant difference.

Results

PMN are essential for effective BCG immunotherapy *in vivo*. Clinical observations support a potential functional role for PMN during BCG immunotherapy of bladder cancer (22–24). We used a well-defined murine preclinical model of orthotopic bladder cancer (11) to test this hypothesis *in vivo* and evaluated the influx of PMN into the bladder wall following intravesical BCG instillation. In this model, C57BL/6 mice were intravesically treated with BCG, and the bladders were subjected to anti-Gr-1 immunohistochemistry to visualize neutrophils. Whereas untreated control bladders are moderately infiltrated by PMN (Fig. 1*A*), BCG-treated tumor-bearing mice show a dense neutrophilic infiltrate (Fig. 1*B*). To determine whether these PMN actually contribute to the antitumor effect mediated by BCG, we compared efficacy of BCG therapy in normal control mice and mice depleted for PMN. Successful depletion of PMN was verified by counting Giemsa-stained blood smears (Fig. 2*A*) and by immunohistology of bladder specimens (data not shown). Reduced PMN counts were observed for up to 11 days. Kaplan-Meier survival analyses show an expected therapeutic benefit of BCG treatment in control mice. Importantly, depletion of PMN abrogated the therapeutic effect of BCG and reduced survival to levels of untreated control mice (hazard ratio, 3.55; 95% confidence interval, 1.42-8.88; Fig. 2*B*; $P = 0.007$). These results show that BCG therapy is ineffective in mice devoid of PMN and suggest an important functional role of PMN for the antitumor efficacy of BCG.

PMN direct T-cell trafficking to bladders of BCG-treated mice. Two potential mechanisms on how PMN could contribute to the antitumor effect of BCG are the following: (*a*) direct antitumor cytotoxicity (29) and (*b*) indirect orchestration and modulation of the subsequent immune response. We tested whether the second mechanism is operative during BCG immunotherapy. At first, we determined whether recruitment of cellular mediators of adaptive immunity, such as dendritic and T cells, depends on the presence of PMN. Mice injected with anti-Gr1 or control antibody were intravesically treated with BCG or PBS. Mice were sacrificed and bladder walls were immunostained for CD11c (expressed on the majority of murine dendritic cells) and CD4 antigen expression. $CD4^+$ T cells are of central importance for effective BCG immunotherapy and represent the predominant immune cell subpopulation in BCG-induced granuloma-like bladder infiltrates (8, 10, 14). They exert crucial accessory functions, such as IFN-γ production or activation of cytotoxic effector lymphocytes, like $CD8^+$ T and NK cells (5, 30). As depicted in Fig. 3*A*, intravesical BCG instillation strongly induced $CD4^+$ T-cell infiltration in normal control mice ($P < 0.05$). At the same time, infiltration with $CD11c^+$ dendritic cells was less pronounced in BCG-treated mice (Fig. 3*B*). Strikingly, BCG-induced influx of $CD4^+$ T cells was completely abolished in mice depleted of PMN. These results clearly suggest that T-cell migration to BCG-treated bladders depends on the presence of PMN *in vivo*.

BCG-activated PMN indirectly induce T-cell chemotaxis. We used an *in vitro* Transwell-based two chamber system to obtain direct evidence for and mechanistic insight into BCG-induced PMN-mediated T-cell migration. In a first experimental setting, we analyzed whether BCG-stimulated PMN directly induce T-cell migration. For this purpose, PMN were stimulated with BCG in the lower compartments of Transwell plates, and T-cell chemotaxis through the separating membranes was assayed. Stimulated monocytes served as a positive control in our system and induced substantial T-cell migration. However, there was clearly no increase in T-cell chemotaxis toward supernatants of stimulated PMN

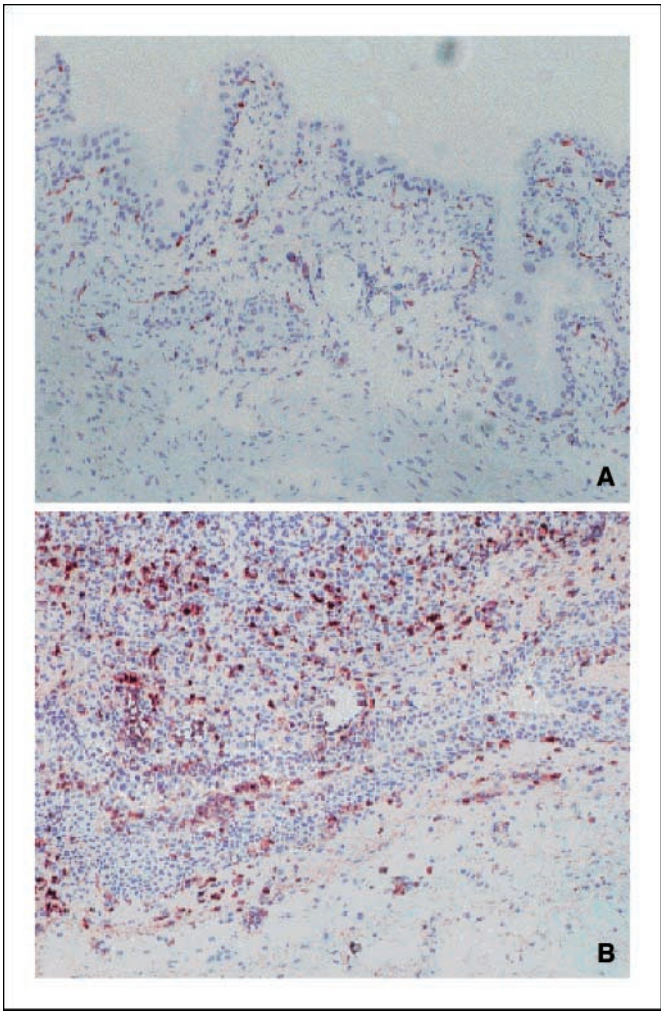


Figure 1. BCG-induced influx of PMN in murine bladder walls. Groups of 6 C57BL/6 mice were intravesically inoculated with 10^5 MB49 bladder tumor cells on day 0 or left without tumor and subsequently treated with intravesical instillations of PBS or $>3 \times 10^6$ CFU BCG on days 1, 8, and 15. On day 16, mice were sacrificed and cryosections of mouse bladders were immunostained for PMN using an anti-Gr1 antibody. Note the moderate neutrophilic infiltrate in PBS-treated mice (*A*) and the dense PMN infiltration in bladders of BCG-treated mice (*B*).

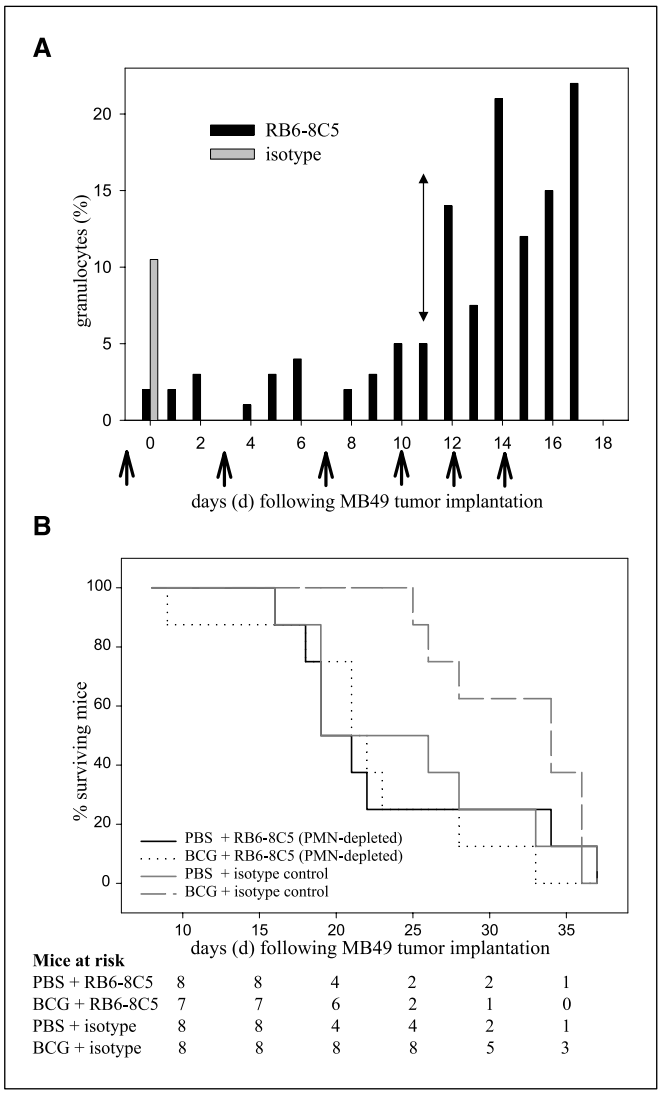


Figure 2. PMN are essential for effective BCG immunotherapy. C57BL/6 mice were intravesically inoculated with 10^5 MB49 bladder tumor cells and received repeated i.p. injections of an anti-Gr1 antibody (RB6-8C5) or the respective isotype control on days −1, 3, 7, 10, 12, and 14. RB6-8C5 treatment (*arrows*) reduced PMN blood counts to <10% of pretreatment levels. *A,* neutropenia persisted for 10 days after which repletion to levels equal to or even greater than pretreatment levels occurred. For immunotherapy, mice were treated intravesically with PBS or BCG (n = 8 per group). *B,* efficacy of BCG therapy is abrogated in neutropenic mice and BCG-treated PMN-depleted mice show a significantly reduced survival compared with BCG-treated control mice (P = 0.007, log-rank test).

compared with culture medium or unstimulated PMN controls even if high amounts of PMN were used (Fig. 4*A*).

We and others have shown previously that the induction of cytotoxic antitumor effector cells during BCG immunotherapy requires a complex interaction of various immune cell subpopulations, including monocytes (5, 9, 30, 31). Monocytes are a potent source of CC chemokines with a strong chemotactic effect on T cells (32, 33). Therefore, we tested whether PMN-induced T-cell migration requires the accessory function of monocytes. First, we investigated monocyte migration toward stimulated PMN (Fig. 4*B*) and then analyzed whether monocytes, after priming in conditioned medium derived from BCG-stimulated PMN, would acquire the potential to induce T-cell migration (Fig. 4*C*). In the first approach, PMN in the lower compartments of Transwell plates were stimulated with BCG and tested for the induction of monocyte chemotaxis. Compared with cell-free culture medium and unstimulated PMN, BCG-activated PMN clearly induced migration of monocytes. Monocyte migration was dependent on the time of PMN stimulation and a 3-hour stimulation period was found to be optimal in this system (Fig. 4*B*). These results show for the first time the direct induction of monocyte chemotaxis following mycobacterial stimulation of PMN.

Although chemokines primarily induce chemotaxis, they additionally synergize with proinflammatory cytokines to activate immune cells, such as monocytes, to undergo functional changes in inflammatory disease (32, 33). We reasoned that BCG-activated PMN in combination with activated monocytes induce T-cell chemotaxis. To test this hypothesis, monocytes were conditioned for 24 hours with the culture supernatant of unstimulated and BCG-stimulated PMN. Thereafter, T-cell migration toward PMN-conditioned monocytes was measured. Interestingly, following priming in culture medium of BCG-stimulated PMN, monocytes induced substantial T-cell migration reaching >50% of the T-cell migration induced by lipopolysaccharide (LPS)–stimulated monocytes. Again, induction of migration was dependent on the time of PMN stimulation with an optimum between 3 and 18 hours

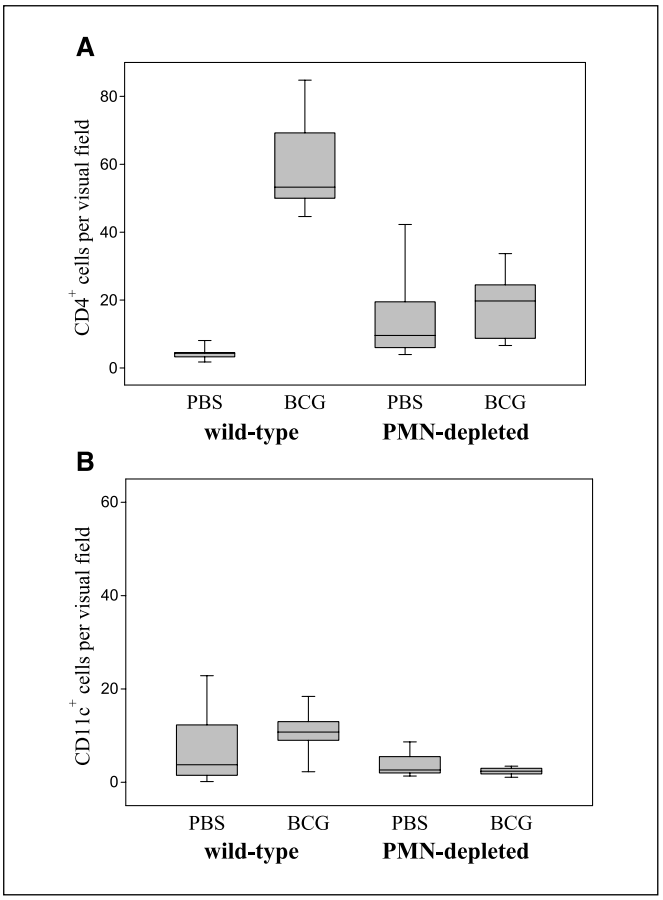
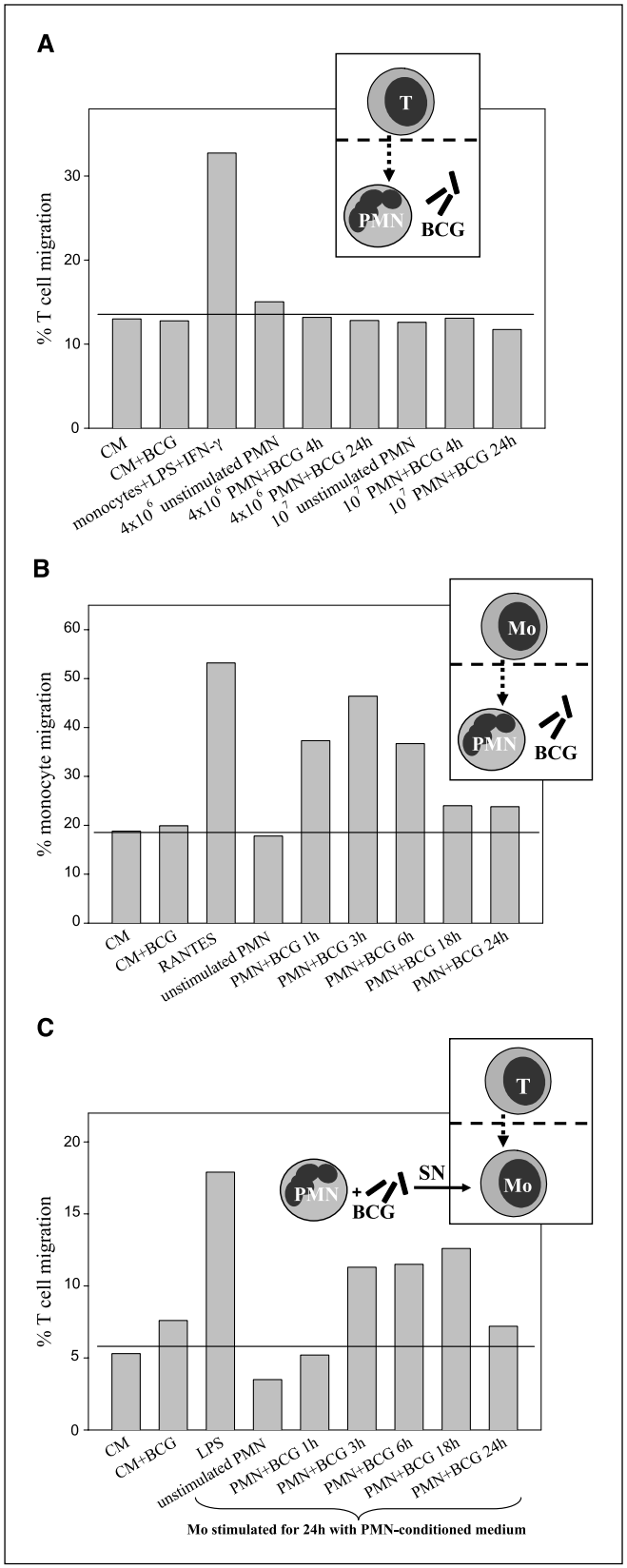


Figure 3. PMN are essential for T-cell trafficking to bladders of BCG-treated mice. Groups of mice (n = 6) each were treated intravesically with BCG or PBS control on days 1 and 8. To deplete PMN, mice received repeated RB6-8C5-injections on days −1, 3, 6, and 8. Control groups were injected with the respective isotype control antibody. Mice were sacrificed on day 9 and bladder walls were immunostained for CD4 (*A*) and CD11c (*B*) antigen expression.

(Fig. 4*C*). To our knowledge, these are the first functional studies providing evidence for the fact that stimulated PMN have the capacity to indirectly induce T-cell chemotaxis via the accessory function of activated monocytes.



BCG-activated peripheral blood PMN and urinary PMN of bladder cancer patients are a major source of inflammatory chemokines and cytokines. In a recent report, we have shown that BCG stimulation prolonged the life span of peripheral blood PMN from healthy donors and induced gene expression of a set of inflammatory and regulatory cytokines and chemokines (20). In the present report, using a preclinical murine model together with *in vitro* migration assays, we have now established an important immunoregulatory role for PMN in directing subsequent local antitumor immune responses during BCG immunotherapy. Although certain chemokines have already been detected in the urine of patients undergoing BCG therapy, the cellular source of these molecules has not yet been identified (4, 22). Consequently, the aim of the final part of our study was to investigate expression of inflammatory chemokines and cytokines by urinary PMN from bladder cancer patients undergoing BCG immunotherapy and to compare this expression with the expression observed in peripheral blood PMN.

Following BCG instillation, PMN are the predominant immune cell population in the urine (9). However, because even small numbers of contaminating monocytes or lymphocytes are likely to also produce chemokines, we first developed a purification protocol to obtain PMN cultures close to homogeneity. This protocol is based on the positive selection of PMN using magnetic bead-coupled antibodies directed against CD66b and lead to the successful depletion of contaminating cells (mostly monocytes) from urinary PMN (Fig. 5*A*) and peripheral blood PMN (Fig. 5*B*).

Subsequent analysis of a broad range of chemokines revealed that the inflammatory chemokines IL-8, GRO-α, and MIP-1α were indeed consistently produced by urinary PMN isolated from patients in the course of BCG immunotherapy. Expression levels were in the same range as expression levels of BCG-stimulated peripheral blood PMN, suggesting substantial *in vivo* activation of urinary PMN (Fig. 5*C*). The chemokines MCP-1, IP-10, RANTES, and MDC were not expressed by urinary PMN and peripheral blood PMN, whereas MIP-1β could be induced in peripheral blood PMN but was absent in urinary PMN (data not shown). Interestingly,

Figure 4. *A,* BCG-stimulated PMN do not directly induce chemotaxis of T cells. PMN (4×10^6 or 10^7) were stimulated with BCG at multiplicity of infection (MOI) = 1 for 4 or 24 hours in complete medium in 24-well plates. Thereafter, Transwells were inserted and T-cell chemotaxis from the upper compartments through the separating 5-μm porous membranes was measured after 2 hours migration time. Unstimulated PMN, complete medium (*CM*), and complete medium with BCG (*CM+BCG*) served as negative controls and supernatants from monocytes stimulated with 100 ng/mL LPS and 2 μg/mL recombinant human IFN-γ (*monocytes+LPS+IFN-γ*) served as a positive control. Only supernatants from stimulated monocytes directly induce T-cell chemotaxis. *B,* BCG-stimulated PMN directly induce monocyte chemotaxis. PMN (4×10^6) were stimulated with BCG (*PMN+BCG*) at MOI = 1 for 1 to 24 hours in complete medium in 24-well plates. Transwells were inserted and monocyte chemotaxis from the upper compartments through the separating membrane was measured after 2 hours. Unstimulated PMN, complete medium, complete medium with BCG were negative controls and 10 nmol/l RANTES served as a positive control. Supernatants from BCG-stimulated PMN induce monocyte chemotaxis with an optimum after 3 hours of stimulation. *C,* the induction of T-cell chemotaxis by BCG-stimulated PMN requires the accessory function of activated monocytes. Monocytes (4×10^6/mL) were incubated for 24 hours in conditioned culture medium generated from unstimulated or BCG-stimulated PMN. For preparation of the conditioned medium, PMN had been stimulated with BCG (MOI = 1) for 1 to 24 hours at 1×10^7/mL. Only monocytes primed in conditioned medium generated from BCG-stimulated PMN induce T-cell chemotaxis with a peak chemotactic activity after 3 to 18 hours of PMN stimulation. Monocytes stimulated with LPS (100 ng/mL) served as a positive control. For (*A-C*), experiments were repeated two to three times each. Representative experiment.

high levels of macrophage migration inhibitory factor (MIF), a still poorly investigated inflammatory cytokine that might play a role in bacterial sepsis and innate immunity to different microbial pathogens, were detectable in both peripheral blood PMN and urinary PMN (Fig. 5*C*).

Discussion

Circulating human PMN are a short-lived subpopulation of innate immune cells with a half-life of only 6 to 10 hours after being released from the bone marrow and undergo rapid apoptosis followed by elimination in the liver and spleen (34). On tissue injury, they home to the site of trauma or infection and migrate from the bloodstream into the inflamed tissue. These activated, infiltrating PMN have a considerably longer life span than their circulating counterparts (35). They can phagocytose microorganisms, generate reactive oxygen intermediates, and release lytic enzymes with antimicrobial potential (21). PMN are the first immune cells to leave the blood vessels and infiltrate tissue that has been invaded by mycobacteria (36). Although recent evidence from *in vivo* studies emphasized the importance of PMN in the immune response to mycobacterial infections, their functional role in this process remains ill defined (15, 16). Similarly, in bladder cancer patients treated with BCG, PMN are the first and most abundant immune cell subpopulation immediately following BCG instillation (8, 9). Yet, until today, they have been largely ignored as playing any role in the BCG-induced antitumor action. Studies by various groups, including ours, showed secretion of a large array of proinflammatory cytokines and chemokines from PMN following mycobacterial stimulation *in vitro* (20, 37, 38). The present study was designed to define a potential functional role of PMN during BCG immunotherapy of bladder cancer. Our results clearly show that PMN are an indispensable immune cell subpopulation for effective BCG therapy. Using the same *in vivo* bladder tumor model, $CD4^+$ and $CD8^+$ T cells as well as NK cells have been identified as the major immune cells mediating the late antitumor effector phase (12–14). We hypothesized that early PMN-derived chemotactic factors are necessary to secondary attract effector cell populations. Following BCG instillation, high levels of proinflammatory cytokines and also chemokines, such as IL-8, can be detected in the urine of treated patients, yet the cellular origin of these mediators had thus far been unknown (5, 22). In the present report, we show *de novo* synthesis of GRO-α as well as an increase in IL-8, MIP-1α, and MIF secretion in activated PMN from the urine of BCG patients and from the peripheral blood of healthy donors. With respect to the vast numbers of PMN in the urine after BCG administration, we reason that these cells are the dominating source of early urinary chemokines, such as IL-8. Interestingly and in contrast to most other cytokines, IL-8 has been shown to appear in the urine already after the first BCG instillation. Additionally, high urinary IL-8 concentrations are associated with the subsequent development of a strong immune response and low disease recurrence rate (22, 39). To our knowledge, GRO-α, MIP-1α, and MIF have thus far not been detected in the urine of

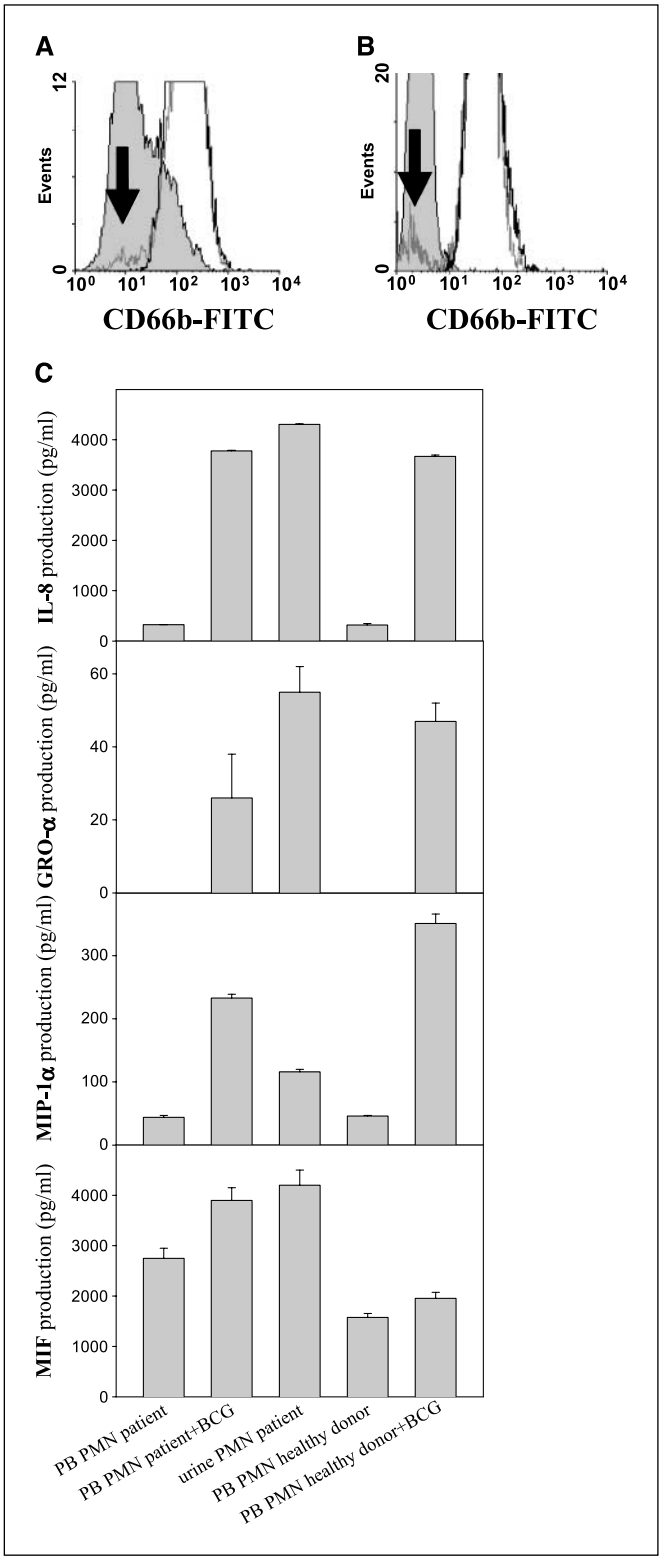


Figure 5. BCG-activated peripheral blood PMN and urine PMN actively secrete proinflammatory chemokines and cytokines. PMN were isolated from the urine (*A*) and the peripheral blood (*B*) of BCG-treated bladder cancer patients. Expression of CD66b on crude (*light gray*) and immunomagnetically purified (*black*) preparations of PMN was analyzed by flow cytometry. Filled histograms are isotype control stainings. Note the disappearance of the contaminating leukocytes (*black arrow*) after purification and the unchanged expression of the granulocyte-specific activation marker CD66b on crude and purified preparations. To analyze chemokine and cytokine expression, urinary PMN of patients and peripheral blood PMN of patients and healthy donors were stimulated overnight with BCG (MOI = 1) or left untreated. *C,* supernatants were collected and tested for chemokine/cytokine secretion by ELISA. The experiment was repeated eight times with similar results. Representative experiment.

BCG-treated patients in larger studies. We now report the active secretion of CXC as well as CC chemokines by BCG-stimulated PMN *in vitro* and *ex vivo* and provide evidence for a functional role of PMN-derived chemotactic factors in monocyte and T-cell migration. Specifically, we can show that BCG-activated PMN directly induce monocyte chemotaxis and indirectly regulate T-cell chemotaxis via the accessory function of monocytes. Thus far, we could not attribute this effect to a single chemokine. However, considering the diverse biological effects of chemokines, they are likely to act in concert (32, 33). Notably, this activity seems to be subject to tightly controlled counterregulation as prolonged PMN stimulation leads to a secondary down-regulation of monocyte and T-cell migration.

Although this is the first functional study to describe monocyte and T-cell migration following mycobacterial stimulation of PMN, there is some evidence concerning T-cell chemotaxis in response to mycobacterial monocyte/macrophage activation. Ferrero et al. (40) reported on the induction of T_H1 cell and γδ-T-cell chemotaxis after pulsing alveolar macrophages isolated from patients with various pulmonary diseases with *Mycobacterium tuberculosis* cell wall components. Interestingly, mycobacterial stimulation of resting monocytes alone was not sufficient, whereas stimulation of preactivated alveolar macrophages induced strong T-cell migration. This is in concordance with our experiments, in which T-cell attraction was due to the combined action of BCG-activated PMN and primed monocytes. Additionally, the crucial role of neutrophils for T-cell attraction was shown *in vivo*. PMN depletion from BCG-treated mice significantly reduced the influx of $CD4^+$ T cells into the bladder wall. We also tried to stain BCG-treated mouse bladders in control and PMN-depleted mice for macrophages. Unfortunately, all antibodies (e.g., anti-Mac-1 and F4/80) that are available to date either display strong cross-reactivity against the rat antibody required for *in vivo* PMN depletion or show coexpression on PMN and macrophages (e.g. CD11b).

PMN depletion from mice with the RB6-8C5 antibody is a commonly used and well-established procedure (25, 41). Recently, however, plasmacytoid IFN-α-producing dendritic cells have been described to express low levels of the Gr-1 antigen together with CD11c (42, 43). Interestingly, despite BCG-induced recruitment of T cells to the bladder, CD11c counts were only marginally induced on BCG stimulation. In anti-Gr-1-treated mice, a minimal reduction in CD11c counts was observed, possibly reflecting a codepletion of Gr-1/CD11c double-positive cells. Although we have not formally addressed the role of $CD11c^+/Gr\text{-}1^+$ cells in our model, these data suggest that $CD4^+$ T-cell recruitment is largely independent of $CD11c^+$ cells and almost exclusively depends on the activity of $CD11c^-/Gr\text{-}1^+$ PMN.

Whereas, with the exception of IL-8 (see above), there are thus far no studies investigating the role of chemokines in BCG immunotherapy, there are some studies addressing the role of chemokines during infections with *M. tuberculosis* in humans. IL-8, GRO-α, and MIP-1α together with MCP-1 and IP-10 are up-regulated in granuloma formation following *M. tuberculosis* infection (40, 44, 45). MIP-1α, MCP-1, and IP-10 are major factors for the chemoattraction of T_H1 and $CD8^+$ T cells, and IL-8, GRO-α, MIP-1α, and MCP-1 are important in monocyte recruitment (32, 33, 40, 44, 45). However, the cellular source of the respective chemokines has thus far not been revealed *in vivo*. Nevertheless, these data support our initial hypothesis that PMN-derived chemokines are important for initiating the BCG-induced immune response by attracting other immune cells to the bladder. Thus, it is tempting to speculate that both in the immune response following *M. tuberculosis* infection and in BCG therapy of cancer BCG-activated PMN might induce a two-step process resulting in direct monocyte and indirect T-cell recruitment.

In addition to IL-8, GRO-α, and MIP-1α, we also detected a significant increase in MIF secretion from BCG-stimulated peripheral blood PMN as well as from urine PMN compared with baseline levels. MIF is an interesting and still somewhat mysterious cytokine with diverse actions within the immunoneuroendocrine system that are yet to be fully understood. Recently, it has been described to serve as a central regulator of innate and inflammatory immune responses. Thus, it controls the "set point" and magnitude of immune reponses by overriding the immunosuppressive effects of glucocorticoids. Even more important, accumulating experimental evidence suggests a crucial role of MIF in the pathogenesis of different inflammatory diseases and in the innate immune response to a broad range of microbial pathogens (46, 47). PMN have only been identified recently as a significant source of MIF in the induction and onset of bacterial sepsis *in vivo* (48). Our data now suggest that PMN-derived MIF might also be involved in the control of the BCG-induced inflammatory antitumor response.

Our identification of PMN as a powerful source of proinflammatory chemokines and cytokines *in vivo* also adds further evidence to the current discussion about the role of PMN in tumor immunology. In advanced malignant diseases, PMN have been described to exert a nursing function on tumor cells by providing growth and proangiogenic factors as well as matrix-degrading enzymes (17, 18). Recently, however, an anticancer potential of PMN during early tumor growth was shown in preclinical animal models (18). In murine models, the application of tumor cells genetically engineered to release various proinflammatory cytokines results in a massive local immune reaction characterized by numerous PMN, which results in the rejection of the tumor cells (49). In these studies, PMN additionally cooperate with antigen-presenting cells and T cells to establish significant antitumor immune memory against the wild-type parental tumor (50). Considering our results, it could well be that the major role of PMN also in those experimental systems lies in the early recruitment and activation of adaptive immune cells, such as T cells.

In summary, we describe for the first time that BCG-stimulated PMN are an indispensable subset of immunoregulatory cells and orchestrate T-cell chemotaxis to the bladder during BCG immunotherapy. Using functional chemotaxis assays, it could be shown that activated PMN additionally induce monocyte migration and exploit the accessory function of these immune cells to attract T cells. In concordance with previous studies, our results provide strong evidence for a mechanism of PMN-mediated tumor cell rejection, in which PMN shape the development of the antitumor immune response by providing early chemotactic and activating factors for subsequent adaptive immunity.

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