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TLR Stimulation of Prostate Tumor Cells Induces Chemokine-Mediated Recruitment of Specific Immune Cell Types

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TLRs boost antimicrobial response mechanisms by epithelial cells and represent the first line of defense at mucosal sites. In view of these immunomodulatory properties, TLR stimulation may represent a novel means to activate anticancer immune responses. In the present study, the ability of TLR ligands to affect the recruitment of different immune cell populations by human prostate cancer cell lines and the underlying mechanisms were investigated. We showed that LNCaP and DU-145 cells express functionally active TLR3 and TLR5. Treatment with their respective agonists, polyinosinic:polycytidylic acid and flagellin, rapidly triggered NF-κB-dependent upregulation of different inflammatory molecules, as assayed by microarray and ELISA. Furthermore, we demonstrated that conditioned media from polyinosinic:polycytidylic acid- and flagellin-treated LNCaP and DU-145 cells induced the recruitment of different leukocyte subpopulations, suggesting that TLR stimulation is able to activate the earliest step of immune response mediated by soluble factors. Interestingly, the more aggressive cancer cell line PC3 expressed TLR3 and TLR5 but failed to respond to TLR agonists in terms of NF-κB activation and the ability to attract immune effectors. Overall, these data show for the first time that TLR3 and TLR5 stimulation of human prostate cancer cells triggers the production of chemokines, which, in turn, favor the attraction of immune effectors, thereby representing a tool to enhance the efficacy of conventional therapies by stimulating anticancer immune responses. The Journal of Immunology, 2010, 184: 6658–6669.

Toll-like receptors are a family of transmembrane receptors that recognize conserved molecular patterns of microbial origin called pathogen-associated molecular patterns. In addition, TLRs play an important role in tissue repair and tissue injury-induced inflammation. In the latter context, TLR ligands can be microbial or host derived (endogenous). TLRs that recognize lipid and protein ligands (TLR1, TLR2, TLR4, TLR5, and TLR6) are expressed on the plasma membrane, whereas TLRs that detect viral nucleic acids (TLR3, TLR7, and TLR9) are localized in lysosomal compartments (1). TLRs transduce signals through different adaptor proteins, which trigger a signaling cascade involving NF-κB, MAPKs, JNKs, p38, and ERKs, and IFN regulatory factors (IRF3, IRF5, and IRF7) (2). Many of the known downstream effects of TLR signaling occur through nuclear translocation of the transcription factor NF-κB and the subsequent production of cell survival and inflammatory molecules, such as TNF-α and IL-1 and -6 (3). The classical TLR function is antimicrobial, consisting of leukocyte recruitment to infected tissues and subsequent induction of adaptive immune responses. Activation of TLRs on epithelial cells leads to surface expression of ICAM-1 (4), which, in endothelial cells, plays a key role in leukocyte rolling and adhesion (5). TLRs were initially thought to be expressed only in immune and epithelial cells, but recent studies demonstrated that these receptors are also expressed in some tumor cells and that such expression is related to tumorigenesis. The role of TLRs in cancer is a matter of debate because conflicting data argue for TLRs being negative or positive regulators of cancer [reviewed in Ref. 6]. The administration of TLR agonists was reported to exert strong antineoplastic effects against established tumors in mice and humans (7). The TLR3 agonist polyinosinic:polycytidylic acid [poly(I:C)] leads to the apoptosis of tumor cells (8, 9), whereas the TLR4 agonist LPS is able to kill ancillary cells of the tumor microenvironment, such as the vascular endothelium (10). TLR activation may also cause tumor regression by increasing vascular permeability and through the recruitment of leukocytes, which determines tumor cell lysis by NK and cytotoxic T cells (6). Accordingly, one of the most promising effects of TLR stimulation by specific agonists in cancer therapy is the activation of the adaptive immune system (7, 11).

In contrast, stimulation of some TLRs was shown to enhance tumor survival and proliferation in vitro (12), suggesting that stimulation of TLRs favors tumorigenesis. The contradictory evidence that, under certain conditions, inflammation promotes carci-
nogenesis, whereas in others it exerts antitumor effects, could be explained by the different intensity and nature of the inflammatory response. In fact, chronic inflammatory processes are milder than acute inflammatory responses, which are aimed at inducing pathogen clearance. In most cases, cancer-associated inflammation is similar to chronic inflammation, including the production of factors that stimulate tissue repair and cancer cell survival and proliferation. However, if the inflammatory response develops into acute inflammation, an innate effector mechanism is activated, and cancer regression takes place (13). Among the different elements that control neoplastic processes, a major role is attributed to members of the chemokine superfamily, a large number of small m. w. proteins that regulate the targeting of leukocytes to inflammatory sites, binding to seven-transmembrane-domain receptors coupled to heterotrimeric G proteins. Chemokines expressed by tumor cells and by host cells play a critical role in determining the fate of the developing tumor by regulating the migration of different leukocyte subtypes (14). The relative proportion of each defense cell type within the tumor (e.g., macrophages, T cells, NK cells, dendritic cells, or other leukocyte subtypes) largely dictates the immune profile at the tumor site; local production of numerous inflammatory mediators is crucial for the recruitment and activation of leukocytes in addition to macrophages and mast cells (15). In particular, CD8 T cells and some types of innate immune cells, such as NK cells, can protect against experimental tumor growth (16).

Prostate cancer (PCa) represents one of the most common cancers diagnosed in males in Western countries. Standard pharmacological therapy, consisting of ablation of androgens, is initially efficacious, but most treated patients develop progressive disease and eventually die of cancer. Consequently, many efforts are being made to identify novel targets and agents useful for the treatment of this disease. Although there is emerging evidence that inflammation might be involved in the etiology of PCa (17), several questions on this issue remain unanswered. In particular, the possible causes of chronic inflammation in the prostate, as well as the involvement of inflammatory cells in the process, have not been fully investigated. Many pathogens, including different bacterial species and viruses, have been detected in the prostate (18, 19), but only some of them are associated with inflammation. Interestingly, many bacterial sequences (20) and a novel viral sequence (21) have been detected in pathological prostate tissue, although the corresponding infective agents could not be isolated and cultured by traditional means. These observations open a new field of investigation aimed at clarifying the possible role of bacterial and viral sequences in the activation of TLRs that might positively or negatively influence tumorigenesis and/or neoplastic progression by triggering the innate and adaptive immune responses. In addition, regardless of the role of TLRs in the initiation of cancer, it is likely that the activation of TLRs in cancer cells influences tumor progression through interaction with the immune system. In this study, we investigated the effects of TLR3 and TLR5 activation on the expression of inflammatory molecules and the subsequent recruitment of different leukocyte populations in LNCaP, DU-145, and PC3 cells, which display low, moderate, and high metastatic potential, respectively (22). Our results suggested that TLR3 and TLR5 stimulation in human PCa cells with different aggressiveness differentially influences innate and adaptive immune responses.

Materials and Methods

**Cell lines and reagents**

LNCaP and PC3 PCa cells were maintained in DMEM, whereas DU-145 cells were maintained in RPMI 1640 medium. Both media were supplemented with 2 mM l-glutamine, 100 IU/ml penicillin-streptomycin, and 10% FCS (Sigma-Aldrich, St Louis, MO). Cells were serum starved for 18 h prior to stimulation with poly(I:C) or flagellin (both from InvivoGen, San Diego, CA) in FCS-free medium. BMS345541 was from Sigma-Aldrich.

**RT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Three micrograms total RNA was used for the RT reaction by using the SuperScript First-Strand Synthesis System Kit (Invitrogen). Each 50 µl PCR mixture contained 1 µl cDNA, 25 pmol specific primers, 0.2 mM 2'-deoxynucleoside 5'-triphosphate mix, 1.5 mM MgCl₂, 5 µl 10X PCR buffer, and 2.5 U Taq DNA polymerase (Invitrogen). For human TLRs and cytokines, the PCR products were amplified using previously reported primers (23–28). The specificity of the primers was tested by a GenBank basic local alignment search tool. A T3 thermocycler PCR system (Bio- metra Whatman, Goettingen, Germany) was used for the reverse transcription reaction, and this was followed by a 30-cycle PCR program.

**Flow cytometry**

LNCaP, PC3, and DU-145 cells were detached with 0.05% trypsin/0.02% EDTA and washed with cold PBS. For detection of TLR5, cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences, Franklin Lakes, NJ), according to the manufacturer’s instructions. Mouse IgG2a anti-human TLR5 (Imgenex, San Diego, CA) mAb or the appropriate isotypic control mAb was used at 0.5 μg/10⁵ cells for 30 min on ice. After washing with cold PBS, cells were stained with fluorescein (FITC)- conjugated anti-mouse (Sigma-Aldrich). Cells were analyzed with a Coulter Epics XL flow cytometer (Beckman Coulter, Brea, CA). Cells were gated using forward versus side scatter to exclude dead cells and debris. Fluorescence of 10⁵ cells per sample was acquired in logarithmic mode for visual inspection of the distributions and in linear mode for quantifying the expression of the relevant molecules by calculating the mean fluorescence intensity.

**Western immunoblotting**

Total LNCaP, PC3, and DU-145 cell lysates were prepared by lysing and scraping the cells off the culture plate with cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing 1 µg/ml leupeptin and 1 mM PMSF (Sigma-Aldrich). Protein concentration was determined by using the micro bicinchoninic acid method (Pierce, Rockford, IL). Equal amounts of proteins (15 µg) were subjected to SDS-PAGE and then transferred onto nitrocellulose. The filters were saturated with 5% nonfat dry milk in TBS. Rabbit polyclonal Ab against total IκB-α was from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific anti-p65 was from BioSource International (Camarillo, CA); Ab against TLR3 was from Cell Signaling Technology, and Ab against α-tubulin was from Sigma-Aldrich. The secondary Abs were HRP-conjugated goat anti-rabbit (Pierce) or goat anti-mouse (Bio-Rad, Hercules, CA). After incubating with the first and secondary Abs, the membranes were washed three times for 15 min with TBS containing 0.1% Tween 20. Ab detection was performed by using the chemiluminescence system (ECL Advance Western blotting detection kit; GE Healthcare Technologies, Milan, Italy).

**Quantification of secreted chemokines by ELISA**

LNCaP cell- or DU-145 cell-conditioned medium (CM) from untreated cells and from cells treated with 100 ng/ml flagellin or 25 µg/ml poly(I:C) (24 µg for assay were used for the presence of CXCL10, CCL3, and CCL5 using the Single Analyte ELISAArray Kit (SA Biosciences, Frederick, MD) and for the presence of IL-8 (R&D Systems Minneapolis, MN), according to the manufacturer’s instructions.

**Luciferase assay**

NF-κB luciferase reporter vector (pNFκB-Luc) was from Stratagene (La Jolla, CA). TLR3-negative (TLR3-DN), a Toll/IL-1R (TLR)-less form of the TLR3 gene generated by deleting the TIR domain (450 bp) (pZERO-hTLR3) was from InvivoGen. One day after plating (1.4 × 10⁶ cells per well) LNCaP cells were cotransfected by means of Lipofectamine 2000 plus reagent (Invitrogen) with pNFκB-Luc and a β-galactosidase vector to normalize for transfection efficiency. In the experiments of TLR3-DN overexpression, LNCaP and DU-145 cells were cotransfected with TLR3-DTIR, pNFκB-Luc, and β-galactosidase vector using Lipofectamine LTX with PLUS reagent (Invitrogen). Transfection was stopped after 5 h by adding DMEM containing 20% FCS. After 18 h, cells were rinsed with fresh medium before stimulation with 100 ng/ml flagellin and 25 µg/ ml poly(I:C) for 6 h followed by lysing using reporter lysis buffer (Promega, Madison, WI). Luciferase activity was assayed with a Berthold luminometer using a luciferase assay kit (Promega), according to the
manufacturer’s instructions. Transfection efficiency was evaluated by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining for each experiment and was always >50%. Data were normalized to protein concentration.

**Microarray**

LNCaP cells were treated for 4 h with 100 ng/ml flagellin or for 2 h with 25 μg/ml poly(I:C); total RNAs from untreated and treated cells were extracted using TRizol reagent (Invitrogen), according to the manufacturer’s instructions. These RNAs were used as a template to generate biotin16-UTP–labeled cRNA probes using the True Labeling Kit (Superarray, Bethesda, MD). The cRNA probes were hybridized at 60°C with the Superarray Human Inflammatory Cytokines and Receptors Microarray membranes, and signals were revealed using the SuperArray Detection Kit. Data from two experiments were analyzed by densitometry using Scanalyze software (Eisen Laboratory, Stanford University, Stanford, CA).

**Chemotaxis assay**

Two types of cell samples from healthy donors were used for in vitro transmigration experiments. In one set of experiments, whole blood deprived of RBCs was used, whereas in another set of experiments, PBMCs obtained by Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden) were depleted of monocytes using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA) to isolate untouched lymphocytes. Briefly, cells were assayed for their ability to migrate through a polyvinylpyrrolidone filter (pore size, 3 μm; Whatman International, Maidstone, U.K.) using Boyden chambers (NeuroProbe, Gaithersburg, MD). In all, 2 × 10^6 cells/well were added to the upper compartment of the chamber, and LNCaP cell or DU-145 cell CM was placed in the lower compartment. In some experiments, PBMCs were pretreated for 18 h with 100 ng/ml Bordetella pertussis toxin (PTx) (Calbiochem, San Diego, CA). The assembled chambers were incubated for 5 h at 37°C. Cells migrated to the lower chamber were counted using a Thoma counting chamber, labeled with previously defined optimal concentrations of mAbs (conjugated with the appropriate fluorochrome), and acquired on a CyAn cytometer (Beckman Coulter). The following Abs were used: CD14 FITC, CD8 FITC, CD19 PE (all from Immunological Sciences, Rome, Italy); CD56 PE, CD16 Pacific Blue (both from BD Biosciences); CD55 ECD, CD16 PE-Cy7, CD56 allophycocyanin (all from Beckman Coulter); CD3 Pacific Blue, CD3 Cascade Yellow, CD4 allophycocyanin (all from DakoCytomation, Glostrup, Denmark); CD19 allophycocyanin-Alexa750, CD54 allophycocyanin-Alexa750 (both from eBioscience, San Diego, CA); CD45RA ECD (Beckman Coulter); and CD25 PE-Cy7 (BD Biosciences). Data were compensated and analyzed using FlowJo software (Tree Star, Ashland, OR). Seven- and eight-color flow cytometry analysis was performed to discriminate among different leukocyte subpopulations: CD3^+ /CD14^-/side scatter high for granulocytes; CD3^+ /CD14^-/side scatter high for monocytes; CD3^-/CD19^+ /CD56^+ /CD16^+ for T cells; CD3^-/CD56^hi/CD16^+ and CD3^-/CD56^lo/CD16^- for cytotoxic-producing and cytotoxic NK cells, respectively; CD3^-/CD4^-/CD52^+ for activated CD4 cells; CD3^-/CD8^-/CD45RA^-/CD16^- for cytotoxic-producing CD8 effector cells and memory CD8 cells; and CD3^-/ CD8^-/CD45RA^-/CD16^- for CD8 effector cytotoxic cells (29).

**Statistical analysis**

Statistical differences were determined by the Student t test for paired samples or by one-way ANOVA followed by the Student t test with the Bonferroni correction. A p value <0.05 was considered significant.

**Results**

**LNCaP and DU-145 cells express functionally active TLR3 and TLR5**

To investigate the expression pattern of TLRs in different PCa cell lines, we initially performed RT-PCR on total RNA. LNCaP and DU-145 cells consistently expressed high levels of TLR3 and TLR5 mRNAs (Fig. 1A). In contrast, the other TLRs tested were absent in both cell lines, with the exception of faint expression of TLR2 in LNCaP cells and TLR6 in DU-145 cells. PC3 cells showed a different pattern of expression, with high expression of TLR2, TLR3, TLR4, TLR5, and TLR6 and no expression of TLR7, TLR8, or TLR9 (Fig. 1A). Next, we tested the expression of TLR3 and TLR5 at the protein level, the only TLRs expressed by the three cell lines tested. We demonstrated by Western blot that TLR3 is expressed at similar levels in LNCaP and DU-145 cells, with a slightly lower expression in PC3 cells (Fig. 1B). Moreover, LNCaP, PC3, and DU-145 cells were stained with a TLR5-specific Ab and analyzed by flow cytometry. As shown in Fig. 1C, TLR5 protein was expressed at high levels in all three cell lines.

TLR stimulation leads to the activation of NF-κB in most cells (30). This transcription factor is retained in the cytoplasm in an inactive form, associated with the inhibitory protein IκBα. TLR agonists induce IκBα phosphorylation and its subsequent degradation by the proteasome, allowing NF-κB to enter the nucleus. Alternatively, NF-κB can be directly activated through the phosphorylation of the NF-κB p65 subunit (31). To study TLR3 and TLR5 activity, LNCaP and DU-145 cells were treated for increasing lengths of time with the respective ligands poly(I:C) (25 μg/ml) and flagellin (100 ng/ml), and NF-κB activation was evaluated. Western blotting of whole lysates showed that in both cell lines, p65 phosphorylation and parallel degradation of IκBα were triggered by poly(I:C) and flagellin stimulation, although with slower p65 phosphorylation kinetics induced by poly(I:C) compared with flagellin (Fig. 2A). In DU-145 cells, p65 basal phosphorylation was much higher than in LNCaP cells, and a less apparent increase was induced by TLR3 and TLR5 stimulation. To confirm activation of NF-κB by poly(I:C) and flagellin, cells were transfected with an NF-κB–dependent luciferase reporter plasmid. The next day, cells were challenged with poly(I:C) or flagellin for 2, 4, or 6 h, and lysates were assayed for NF-κB–dependent luciferase activity. Poly(I:C) and flagellin induced a significant NF-κB acti-
vations, albeit with different kinetics (Fig. 2B). In LNCaP cells, the highest level of enhancement was reached as early as 2 h after flagellin treatment, whereas 4 h were required for maximal activation by poly(I:C). In DU-145 cells, the kinetics of NF-κB activation were delayed, with the maximum increase in luciferase activity detectable after 6 h of poly(I:C) or flagellin stimulation.

PC3 cells showed a strong basal p65 phosphorylation, with no additional induction by poly(I:C) or flagellin. Moreover, NF-κB-dependent luciferase activity in this cell line was also basally high, and it unexpectedly decreased after stimulation with flagellin. Altogether, these data demonstrate that TLR3 and TLR5 trigger activation of NF-κB in LNCaP and DU-145 cells.

**Poly(I:C)-triggered NF-κB activation is TLR3 dependent**

Exogenous flagellin can interact only with membrane-bound TLR5, whereas different molecules are involved in dsRNA recognition. In fact, poly(I:C) is able to bind TLR3 localized in the endosomal membrane, but it can also activate different pathways mediated by cytosolic sensors (32, 33). To determine the direct involvement of TLR3 in mediating poly(I:C)-triggered effects, LNCaP and DU-145 cells were transiently transfected with a control plasmid or with a vector encoding TLR3-DN (nonfunctional because of deletion of the TIR domain), which competes with the endogenous functional TLR3. Transfected cells were treated for 4 h with poly(I:C) and analyzed for NF-κB activation by luciferase assay. We found that downregulation of TLR3 function by TLR3-DN overexpression significantly reduced poly(I:C)-induced NF-κB activation in both cell lines (Fig. 3).

**TLR3 and TLR5 stimulation leads to differential secretion of chemokines**

NF-κB is a key regulator of TLR-induced proinflammatory molecules (34, 35). Thus, we sought to analyze whether TLR3/5 ligands induce the production of chemokines in LNCaP and DU-145 cells through the NF-κB pathway. To this end, we performed ELISA on CM from LNCaP and DU-145 cells treated for 24 h with poly(I:C) or flagellin. None of the chemokines tested was detectable in control LNCaP CM, whereas DU-145 cells constitutively secreted low levels of CCL5 and greater amounts of IL-8 (Fig. 4). Our results indicated that in both cell lines, poly(I:C) was more effective than flagellin in the induction of CCL3, CCL5, and CXCL10, and only in DU-145 cells did both TLR agonists elicit IL-8 production at the same levels (Fig. 4).

**LNCaP and DU-145 cells stimulated with poly(I:C) and flagellin recruit different leukocyte subpopulations**

The chemokine superfamily plays a major role in the control of neoplastic processes by regulating the directed trafficking of leukocytes to inflammatory sites and enabling their recruitment from hematopoietic organs (36–38). The activity of chemokines can exert significant support or inhibition of processes involved in tumorigenesis, depending on the malignancy context (14). To clarify the possible functional role of the chemokines upregulated by treatment with poly(I:C) or flagellin, we performed a chemotaxis assay using Boyden chambers and evaluated the recruitment of different immune effector cells across a filter (pore size, 3 μm). The chamber below the filter was filled with CM from LNCaP or DU-145 cells stimulated with poly(I:C) or flagellin, and total human leukocytes from healthy blood donors (PBMCs) were plated in the upper chamber. After 5 h at 37˚C, the cells migrated through the filter to the lower chamber were counted and stained with Abs directed to specific surface Ags to discriminate among different leukocyte subpopulations (see Materials and Methods). As shown in Fig. 5A, poly(I:C) CM and flagellin CM from LNCaP cells induced a 2-fold increase in the chemotaxis of granulocytes and monocytes versus control CM. However, only poly(I:C) CM enhanced the recruitment of T and B lymphocytes and NK cells compared with control CM. Poly(I:C) CM and flagellin CM from DU-145 cells...
from three independent experiments. Data represent the mean of triplicate samples poly(I:C)-stimulated CMV-transfected cells with poly(I:C)-stimulated versus untreated samples. Statistical analysis was performed by comparing efficiency. Data are expressed as fold of induction in TLR agonist-treated assay.

Subsequently, cells were treated or not with poly(I:C) for 4 h prior to luciferase transiently transfected with CMV (control plasmid) or TLR3-DN. Sub-DU-145 cell lines transfected with TLR3-DN plasmid. Both cell lines were role in antitumor immune defense, we set out to characterize the of TLR-induced chemokines produced by LNCaP cells, the most reduced by TLR stimulation (Fig. 5C). We observed that toxin pretreatment blocked the effect induced by TLR stimulation (Fig. 2). Because chemokines bind to seven-transmembrane-domain receptors that are coupled to heterotrimeric G proteins, chemokine-induced migration is completely inhibited by treatment of the cells with PTH, which inhibits Ga and G proteins (39). To confirm the nature of the chemotactic activity induced by TLR agonists, we pretreated total leukocytes with PTH for 18 h before testing their overall migration in TLR-stimulated LNCaP CM. We observed that toxin pretreatment blocked the effect induced by TLR stimulation (Fig. 5C), demonstrating the direct role of TLR-induced chemokines produced by LNCaP cells, the most TLR-responsive cell line, on leukocyte recruitment.

Subsequently, because T lymphocytes and NK cells have a key role in antitumor immune defense, we set out to characterize the subpopulations of lymphocytes and NK cells that are selectively recruited by TLR-stimulated LNCaP and DU-145 cells. To this end, PBLs from healthy donors were isolated by Ficoll gradient and magnetic depletion of the monocyte population (see Materials and Methods) and subjected to chemotaxis assay.

Preliminarily, because it is well known that flagellin and poly (I:C) affect various functions of lymphocytes and NK cells (40, 41), we assayed DMEM + 25 μg/ml poly(I:C) and DMEM + 100 ng/ml flagellin, incubated 24 h at 37°C, for their chemotactic activity. Direct treatment of the PBLs with flagellin or poly(I:C) did not enhance chemotaxis (data not shown).

As for LNCaP cells, flagellin CM had no effect on lymphocyte migration, whereas poly(I:C) CM from LNCaP cells induced the migration ~70% of the total CD4+ T lymphocytes (Fig. 6, upper left panel), the most represented T cell subset in the upper chamber, with a 3-fold increase compared with control CM. For the less represented subsets, such as activated CD4+ T cells and CD4+CD25high T cells (regulatory T cells), poly(I:C) CM induced an increase in their migration, although the absolute number of regulatory T cells was very low. As for CD4+ T lymphocyte migration with DU-145 CM, poly(I:C) CM, and flagellin CM, they all induced a significant increase in migration compared with the very low rate observed with control CM (Fig. 6, upper right panel). With regard to total CD8+ T cells, 83% were attracted by LNCaP poly(I:C) CM (Fig. 6, middle left panel). A detailed analysis of the migrated CD8+ cells showed that the total fraction of the major cytotoxic population of CD8+ T cells and 73% of memory cytokine-producing cells were attracted by poly(I:C) CM (Fig. 6, middle left panel). Interestingly, 100% of NK lymphocytes, both cytokine producing and cytotoxic cells, were recruited by poly(I:C) CM (Fig. 6, lower left panel).

With regard to CM from DU-145 cells, flagellin and poly(I:C) CM induced a significant recruitment of total CD8+ memory cytokine-producing T and NK cells (Fig. 6, middle and lower right panels); however, the rate of migration of T and NK cell subpopulations was less than that induced by CM from untreated and TLR-stimulated LNCaP cells.

**TLR3 and TLR5 stimulation induces expression of a proinflammatory mRNA array in LNCaP cells**

Because the strongest chemotactic effect was obtained by using LNCaP CM, we tested the effect of flagellin and poly(I:C) on a broad array of cytokines and chemokines by Microarray analysis in this cell line. RNA extracted from cells stimulated with TLR3 or TLR5 agonists was hybridized onto chips containing a subset of 113 key genes involved in the inflammatory response. LNCaP cells were treated with poly(I:C) for 4 h and flagellin for 2 h when NF-kB activation by these agonists reached its peak (Fig. 2B). Table I and Fig. 7B show that poly(I:C) and flagellin caused a marked change in the gene-expression pattern; 26.7% and 15%, respectively, of tested genes were upregulated. Data obtained for all of the genes tested are shown in Supplemental Fig. 1. Chemokines belonging to the CC and CXC subfamilies, which account for 52% of the upregulated genes, showed a similar pattern of expression following stimulation with both agonists. However, a number of functionally different chemokines were selectively induced by poly(I:C) or flagellin (Table I). Moreover, poly(I:C) induced the expression of several other inflammatory molecules, such as the cytokines IL-6 and -15, TNF, linotoxin α, and linotoxin β (LTB); the cytokine receptors IL-10RA, -13RA2, and -15RA; the chemokine receptor CXCR4; the antiviral molecule IFN-A2; the complement component C3; and the transcription factor CCAAT/ enhancer binding protein β, whereas flagellin enhanced only CXCR4, IL-15 and -15RA, LTB, and TNF.0.05; **p ≤ 0.01, Student paired t test.
To determine whether these effects on gene expression were dependent on NF-κB activity, we used the specific IKK inhibitor BMS345541 (42), which efficiently blocked NF-κB activation induced by both TLR agonists in reporter assays (Fig. 7A). Cells were pretreated 1 h with the inhibitor (10 μM) before stimulation with poly(I:C) for 4 h prior to total RNA extraction. As shown in Fig. 7B and Supplemental Fig. 1, pretreatment with BMS345541 dramatically suppressed the expression of inflammatory genes induced by poly(I:C), indicating a direct involvement of NF-κB in their upregulation. The same degree of inhibition was observed on the flagellin-induced genes tested by RT-PCR (data not shown).

To confirm the microarray data, upregulation of some key inflammatory cytokines and chemokines after flagellin and poly(I:C) treatment was assayed by RT-PCR. In agreement with results obtained by microarray, we observed that both TLR agonists induced a strong increase in IL-8 and TNF-α expression, whereas only poly(I:C) upregulated IL-6 and CXCL9 mRNA (Fig. 7C).

Discussion

TLRs play an important role in cancer development, a concept supported by the association of numerous polymorphisms in TLRs with human cancer in a variety of organs, including the prostate (43). In this study, we investigated the expression of TLRs in different human PCa cell lines (LNCaP, DU-145, and PC3) and showed that only TLR3 and TLR5 are shared by the three cell lines. Moreover, we demonstrated, for the first time in prostate tumor cells, that ligand-induced activation of TLR3 and TLR5 leads to selective migration of leukocytes through upregulation...
of different chemokine patterns. Several recent reports on the expression of TLRs in human tumors, including prostate carcinomas (44), suggested conflicting roles for the function of TLRs in human cancer (45–47). Infection- or injury-induced inflammation can promote tumorigenesis, owing to chronic tissue damage with subsequent tissue repair that could evolve in

FIGURE 5. LNCaP and DU-145 cells stimulated with poly(I:C) and flagellin produce chemokines that induce differential leukocyte chemotaxis. LNCaP (A) or DU-145 (B) cells were stimulated with poly(I:C) (25 μg/ml) or flagellin (100 ng/ml). After 24 h, CM were collected from stimulated and unstimulated (Ctr) cells and used for a chemotaxis assay (using Boyden transwell cell culture chambers) on total leukocytes from healthy blood donors. Migrated human leukocytes were collected, labeled with Abs recognizing leukocyte subpopulations, and analyzed by flow cytometry. Significant differences in the leukocyte migration rates induced by poly(I:C) CM or flagellin CM (versus Ctr CM) are shown. ANOVA with Bonferroni posttest. C. Migration of total leukocytes pretreated or not for 16 h with PTx (100 ng/ml) prior to stimulation with CM from untreated LNCaP cells or TLR agonist-treated LNCaP cells. Statistical analysis was performed by comparing chemotactic response in the presence versus the absence of PTx in poly(I:C) and flagellin CMs. Data represent the mean ± SEM of a quadruplicate from a representative experiment, which was replicated three times. *p ≤ 0.05; **p ≤ 0.01, ANOVA with Bonferroni posttest.
uncontrolled cell proliferation. On the other hand, cancer growth can mimic tissue damage, and this can trigger TLR-dependent production of numerous inflammatory mediators associated with the recruitment and activation of leukocytes, particularly mast cells, macrophages, and neutrophils with tumor-promoting properties (48). Conversely, TLR agonists were shown to directly kill tumor cells or to cause tumors to regress indirectly by recruiting NK and cytotoxic T cells (6). In this article, we demonstrated that stimulation of TLR3 and TLR5 significantly upregulated chemokines and other inflammatory molecules in LNCaP and DU-145 cells, but not in PC3 cells (data not shown). Importantly, the induction of such factors determines oriented migration of different immune cell subpopulations, suggesting that TLR stimulation is able to activate the earliest step of immune response mediated by soluble factors in the prostate. Our data showed that LNCaP cells stimulated with both TLR agonists triggered chemotaxis of T lymphocytes and NK cells much more effectively than did DU-145 cells, whereas granulocyte migration was induced by both cell lines at the same extent. On the contrary, CM from PC3 cells stimulated with poly(I:C) or flagellin did not enhance chemotaxis of leukocyte subpopulations. We also demonstrated that the chemotactic effect of TLR-stimulated media depended on chemokines, because pretreatment of PBMCs with PTx, which specifically inhibits G protein-mediated signaling and, thus, chemokine effects, blocked the migration of leukocytes. These processes depend on the NF-κB pathway because pretreatment with a specific inhibitor completely abolished the upregulation of inflammatory cytokines and chemokines induced by TLR stimulation. These results are in line with the proposed role of NF-κB in the regulation of the inflammatory process in innate and adaptive immune responses, through the local production of proinflammatory factors (49). Noteworthy, in PC3 cells, NF-κB1 is constitutively activated at high levels and was not induced further by poly(I:C) or flagellin treatment, consistent with the lack of

FIGURE 6. Different lymphocyte subpopulations are attracted by LNCaP or DU-145 CM. PBLs and NK cells from healthy blood donors were isolated by granulocyte and monocyte depletion. T lymphocytes and NK cells were used for chemotaxis assay with CM from LNCaP or DU-145 cells treated or not with 25 μg/ml poly(I:C) or 100 ng/ml flagellin. The total number of cells plated in Boyden chambers (white boxes) and cells migrated after 5 h at 37°C (other boxes) were labeled with Abs against markers specific for NK and T subpopulations, as described in Materials and Methods, and analyzed by flow cytometry. Values are from a representative experiment that was repeated three times. Each value represents the mean ± SEM of a quadruplicate experiment. Statistical analysis was performed by comparing the chemotactic response induced by control CM versus TLR-stimulated CM. *p ≤ 0.05, ANOVA with Bonferroni posttest.
immunity and elicits potent antitumor activity. To our knowledge, this is the first demonstration that the PCa cells LNCaP and DU-145 express TLR5, suggesting that this receptor might be exploited as a therapeutic target. In this regard, the potential role of flagellin in activating an immune response in cancer immunotherapy is under investigation in melanoma and renal carcinoma (53).

In agreement with the multifaceted effects of TLR activation, our results in LNCaP cells demonstrated that the TLR3 agonist poly(I:C) induced the expression of a pattern of cytokines and chemokines different from that induced by flagellin treatment. In addition to the upregulation of chemokines involved in neutrophil and monocyte recruitment, we report that poly(I:C) treatment induced the upregulation of the chemokines CCL3, CCL4, CCL5, CCL8, CXCL9, and CXCL10, specifically interacting with chemokine receptors CCR1, CCR4, CCR5, and CXCR3, which were expressed by freshly isolated primary NK cells and CD8 T cells (54, 55). In accordance with this chemokine panel, such interaction in our model induced massive NK and CD8 T cell chemotaxis. The importance of CXCR3 expression in NK cells and CTLs was recently highlighted by the observation that the therapeutic effect of immunochemotherapy is abrogated in Cxcr3−/− mice (56). In fact, CD8 cytotoxic T cells and NK cells can play an important role in antitumor defenses (57, 58). Recent studies showed that NK cell activity is controlled through a balance between inhibitory and stimulatory receptor signals. One of the activating NK and CD8 TCRs, the NKG2D receptor, may have the ability to specifically target tumor cells for killing through recognition of its ligands, which include the MHC class I chain-related A and B molecules (59). Interestingly, LNCaP cells display high expression

Table I. Upregulation in gene expression induced by poly(I:C) and flagellin in LNCaP cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Poly(I:C) versus Control</th>
<th>Flagellin versus Control</th>
<th>Key Immune Target Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokines</td>
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<tr>
<td>Upregulated</td>
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<td></td>
<td></td>
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<tr>
<td>CCL2</td>
<td>25.8</td>
<td>26.1</td>
<td>Monocytes, mast cells</td>
</tr>
<tr>
<td>CCL20</td>
<td>__</td>
<td>__</td>
<td>Th17, dendritic cells</td>
</tr>
<tr>
<td>CCL3</td>
<td>__</td>
<td>__</td>
<td>CD8 T cells, NK cells</td>
</tr>
<tr>
<td>CCL4</td>
<td>_</td>
<td>_</td>
<td>CD8 T cells, monocytes, NK cells</td>
</tr>
<tr>
<td>CCL5</td>
<td>5.6</td>
<td></td>
<td>Monocytes, NK cells</td>
</tr>
<tr>
<td>CCL8</td>
<td>12.6</td>
<td></td>
<td>Th1 cells, naive and cytotoxic CD8 T cells</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>26.5</td>
<td>14.2</td>
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</tr>
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<td>CXCL10</td>
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<td>__</td>
<td>Neutrophils</td>
</tr>
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<td>__</td>
<td>__</td>
<td>Neutrophils</td>
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<tr>
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<td>Neutrophils, NK cells</td>
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<td>Other Inflammatory Molecules</td>
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<td>Transcription factor</td>
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<td>Malignant cell growth</td>
</tr>
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<td>CXCR4</td>
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<td>Induction of apoptosis; inflammatory response</td>
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<td>IFNA2</td>
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<td>Integral to membrane: receptor activity</td>
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<td>IL10RA</td>
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<tr>
<td>IL6</td>
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<td>__</td>
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<tr>
<td>LTA</td>
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<td>Immune response; induction of apoptosis</td>
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<tr>
<td>TNF</td>
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<td>5.7</td>
<td>Inflammation; antiapoptotic response; tumor progression</td>
</tr>
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</table>

*Ratio of the mean expression between control and poly(I:C) or flagellin samples of two independent experiments.

*Fold changes could not be determined because cDNA hybridization signal was undetectable in control arrays but was clearly detectable in poly(I:C) and flagellin arrays.

CEBPβ, CCAAT/enhancer binding protein β; LTA, linfotoxin α.
of MHC class I chain-related A and B molecules (60), suggesting that the increased recruitment of NK and CD8 T cells as a result of poly(I:C) stimulation may play a part in activating these immune cells. The importance of NK activity in PCa was recently demonstrated in TRAMP mice, an in vivo model of PCa (61). Indeed, we previously demonstrated that poly(I:C) exerts direct anti-proliferative and apoptotic effects on LNCaP cells after 24–48 h of stimulation (8), a rather slow kinetics compared with the very early onset of the upregulation of inflammatory molecules. These two kinetically different effects of poly(I:C) on LNCaP cells converge toward an antineoplastic final effect. By exerting a direct proapoptotic effect on LNCaP cells (8) and an immune-mediated effect due to the recruitment of NK cells and cytotoxic CD8 cells (present data), poly(I:C) is a potentially valid therapeutic agent in PCa, likely capable of improving conventional therapies because it may fulfill different criteria that were recently proposed (62).

It was recently demonstrated in vivo murine tumor models that the TLR3 agonist polyadenylic acid–polyuridylic acid induces the production of CCL5 and CXCL10 (CXCR3 ligand) in the tumor parenchyma. Moreover, CCL5 blockade improved the efficacy of immunochemo-therapy [vaccination followed by conventional chemotherapy in combination with polyadenylic acid–polyuridylic acid injection], whereas CXCR3 blockade abolished its beneficial effects, indicating that TLR3 stimulation can induce tumor cells to produce a range of chemokines that reinforce immunostimulatory or immunosuppressive effect, thus influencing the therapeutic response (56). Accordingly, our data show for the first time that following TLR3 and TLR5 stimulation, human PCa cells produce chemokines with potentially opposite effects on immune system modulation.

The challenge for further investigation will be to exploit poly(I:C) and flagellin in vivo experiments to clarify the consequences of immune cell subpopulation recruitment over neoplastic progression in PCa. Intriguingly, the exploitation of TLRs for cancer immunotherapy and vaccines is promising; in particular, a clinical trial for the treatment of ovarian cancer is in progress exploiting poly(I:C) as an adjuvant together with a vaccine (63).

Altogether, our data indicated that TLR3 and TLR5 were expressed and functionally active in LNCaP and DU-145 PCa cells, whereas TLR3/5 stimulation did not affect NF-κB activation, cytokine/chemokine expression, or immune cell recruitment in PC3 cells. This suggests that PC3 cells, which represent the most aggressive castration-resistant stage of prostate malignancy, are not able to activate an antitumor immune response. In conclusion, the ability to mount an immune response following TLR stimulation seems to be inversely correlated with neoplastic progression.

**FIGURE 7.** Poly(I:C) and flagellin induce differential mRNA expression of proinflammatory molecules via NF-κB activation. A, LNCaP cells were cotransfected with pNF-κB-Luc reporter and β-galactosidase vectors, and the next day they were stimulated for 4 h with 25 μg/ml poly(I:C) or 100 ng/ml flagellin with or without 10 μM BMS345541 pretreatment. Subsequently, cell lysates were subjected to luciferase assay. Statistical analysis was performed by comparing luciferase activity of TLR-stimulated cells with BMS345541-pretreated TLR-stimulated cells. **p < 0.01, Student paired t test.** Transfection efficiency was evaluated by β-galactosidase assay. Data ± SEM represent the mean of triplicate samples from three independent experiments. B, Total RNA from TLR agonist-stimulated LNCaP cells, pretreated or not with BMS345541 for 4 h, were labeled and analyzed on the Superarray Human Inflammatory Cytokines and Receptors chip. Examples of five genes modulated by poly(I:C) or flagellin with or without BMS345541 treatment are circled. C, LNCaP cells were treated with 25 μg/ml poly(I:C) or 100 ng/ml flagellin for 4 and 2 h, respectively. Total RNAs were subjected to RT-PCR using specific primers for IL-6 (462 bp), IL-8 (494 bp), TNF-α (166 bp), and CXCL9 (168 bp). Actin (350 bp) was used as a loading control. Results are representative of three independent experiments.
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Disclosures
The authors have no financial conflicts of interest.

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