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Predominant Expression of CCL2 at the Tumor Site of Prostate Cancer Patients Directs a Selective Loss of Immunological Tolerance to CCL2 That Could Be Amplified in a Beneficial Manner

Liat Izhak,* Gizi Wildbaum,* Weinberg Uri,* Yuval Shaked,† Jennifer Alami,‡ Daniel Dumont,‡ Avi Stein,§ and Nathan Karin*

We have previously shown that, during inflammatory autoimmune diseases in humans, the immune system develops a neutralizing auto-Ab–based response to a very limited number of inflammatory mediators, and that amplification of each response could be beneficial for the host. Our working hypothesis has been that this selective breakdown of immunological tolerance is due to a predominant expression of an inflammatory mediator at an immune-restricted site undergoing a destructive process. All three conditions also take place in cancer diseases. In this study, we delineate this hypothesis for the first time in a human cancer disease and then explore its clinical implications. We show that in primary tumor sections of prostate cancer subjects, CCL2 is predominantly expressed at the tumor site over other chemokines that have been associated with tumor development, including: CXCL12, CXCL10, CXCL8, CCL3, and CCL5. Subsequently, the immune response selectivity mounts an Ab-based response to CCL2. These Abs are neutralizing Abs. These findings hold diagnostic and therapeutic implications. The current diagnosis of prostate cancer is based on prostate-specific Ag measurements that do not distinguish benign hypertrophy from malignancy. We show in this study that development of anti-CCL2 Abs is selective to the malignant stage. From a clinically oriented perspective, we show, in an experimental model of the disease, that DNA-based amplification of this response suppresses disease, which has implications for a novel way of therapy in humans. The Journal of Immunology, 2010, 184: 1092–1101.

In previous studies that were initiated in experimental models of different inflammatory autoimmune diseases and then extended to humans, we have shown that in the course of inflammatory autoimmune diseases, the immune system selectively generates an auto-Ab response to a few inflammatory mediators, mostly chemokines and cytokines, which are thought to participate in promoting the inflammatory process (1–6). For example, we showed that patients suffering from rheumatoid arthritis (RA) but not osteoarthritis display a significant level of neutralizing auto-Ab directed against TNF-α (3). These patients did not mount any auto-Ab response either to several key inflammatory chemokines or to regulatory mediators, such as IL-10 or TGF-β, or even the chemokine CXCL12, which also functions as a regulatory mediator that selects Ag-specific IL-10–producing CD4+ T cells (7). Complementary experiments suggested that in experimentally induced RA, these anti–TNF-α auto-Ab participate in the natural regulation of disease and restrain—although they are incapable of totally preventing—it development (3). Nevertheless, their selective amplification by targeted DNA vaccines led to rapid recovery from an ongoing disease (8). These studies also showed that selective breakdown of tolerance to TNF-α is due to its preferential expression at a partially immune-restricted site undergoing a destructive process (3, 8). Very recently, we have shown that type I diabetes mellitus (T1DM) patients preferentially display auto-Ab production to CCL3 and not to several other proinflammatory chemokines (9). It is yet to be proven that this chemokine dominates the chemokine expression at the autoimmune site. Nevertheless, a previous study showing that selective neutralization of CCL3 suppresses T1DM in NOD mice has implications for the important role of this chemokine in this disease (10).

Similarly to organ-specific autoimmunity in various cancer diseases, one of which is cancer of the prostate (CaP), key inflammatory chemokines are expressed at the primary tumor site, which also undergoes a destructive process at a restricted site. This motivated us to extend our research to CaP. Several recent studies have shown that chemokines, in particular CXCL12 (SDF-1α), CXCL8 (IL-8), CCL2 (MCP-1), CCL3, (MIP-1α), CCL5 (RANTES), and CXCL10 (IP-10), are produced at the tumor site by the CaP cells that also express their receptors, and commonly also by the supporting tissue (11–17). These chemokines are likely to promote tumor development and angiogenesis (11–22).

The current study shows that, of these chemokines, CCL2 is dominantly expressed at the human primary tumor site and that these patients selectively mount an auto-Ab response of neutralizing Abs against CCL2. An immunocompetent model of the disease is then used to explore the consequences of these findings.
Materials and Methods

Specimens and Ab titers in human sera

The human experimental work was conducted together with the Department of Urology at Carmel Medical Center in Haifa, Israel. All sera and tissue samples were obtained from patients according to Helsinki Committee Approval 0038-07-CMC, dated April 16, 2008. Clinical data of the patients are summarized in Table I. Log2 Ab titer against each detected chemokine was measured using an ELISA test on sequential sera dilution, as described in detail below and in (3). All recombinant human chemokines were purchased from R&D Systems (Minneapolis, MN).

Cell lines

All cell lines (PC-3, RAW, TRAMP-C1, and TRAMP-C3) were obtained from the American Type Culture Collection (Rockville, MD).

In vitro chemotaxis assays

Chemotaxis assays of RAW cells (1 × 105) were performed in a TransWell system (5-μm pore size, Corning Costar Corporation, Cambridge, MA) (23). Chemotaxis assays of PC-3 and TRAMP-C1 cell lines (1 × 106) were conducted using the CytoSelect TM cell migration assay (8-μm pore size) (Cell Biolabs, San Diego, CA) (23).

Evaluation of the Ab titer in sera samples

The titer of chemokine-specific Abs in the sera of CaP patients and mice injected with TRAMP-C1 cells was determined using a direct ELISA and by using two complementary measurement methods: comparing the OD measured in wells coated with the appropriate chemokine with those not coated with this recombinant protein when sera were added in serial dilutions from 26–230 (log2 Abs titer), or direct measuring of OD after a single dilution of 1:500 (9). All recombinant chemokines were purchased from R&D Systems.

Cyanogen bromide purification of chemokine-specific Abs

Five milligrams recombinant chemokines (hCCl2, mCCl2) were bound to a cyanogen bromide (CNBr)-activated Sepharose column according to the manufacturer’s instructions (Pharmacia Biotech, Uppsala, Sweden, catalog number 17-0820-01). Specific Abs from sera (1g Fraction) were loaded onto the column and eluted by an acidic elution buffer (glycine pH 2.5).

Chemokine detection by ELISA

Detection of mCCl2 was performed by the ELISA development kit (mouse CCL2/JE ELISA kit, R&D Systems) and conducted according to the manufacturer’s instructions.

Animal models

C57BL/6 male mice were purchased from Harlen (Jerusalem, Israel) and maintained in individual ventilated cages under pathogen-free conditions at the animal facility of the Rapport Faculty of Medicine (Technion). Six-week-old mice were injected s.c. between the two flanks with 7 × 106 TRAMP cells. Tumor diameters were measured using a caliper. Tumor volume was calculated using the formula π/6 × a × b2, where a is the longest dimension and b is the width. TRAMP mice (24) were bred and maintained under pathogen-free conditions at the animal facility of the University of Toronto, Toronto, Ontario, Canada. Results obtained from these mice were also verified (only weeks 8 and 10) using sera from a smaller colony of TRAMP mice obtained from the Animal Facility of the Weizmann Institute (Rehovot, Israel).

Immunohistochemistry and immunofluorescence

All immunohistochemistry and immunofluorescence staining and analyses were conducted according to protocols we previously described (25, 26). For immunohistochemistry, the following Abs were used as primary Abs: polyclonal rabbit antivascular endothelial growth factor (VEGF) (sc-152, Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal rat anti-mouse F4/80 (MCA497B, Serotec, Raleigh, NC) for macrophages. For immunofluorescence as primary Abs, we used mouse anti-hCCl2 (Santa Cruz Biotechnology), mouse anti-hCCl2 (R&D Systems), rabbit anti-hCCl8 (PeproTech, Rocky Hill, NJ), rabbit anti-hCCl10 (PeproTech), and rabbit anti-hCCl3 (PeproTech) all at 1:100 dilutions.

In vitro proliferation assay

In vitro proliferation assay of TRAMP-C1 cells was conducted as we described in detail in (23).

DNA vaccination

The cDNA encoding mouse CCL2 and β-actin were obtained from splenocytes of naive mice and subjected to RT-PCR amplification of the CCL2 and the β-actin–encoding genes using the oligonucleotide primers (forward 5′- ATTGATGCTCCAGACACCTTCTT-3′; reverse 5′-TCAATTTTCATTGTTTGGTG-3′) and β-actin (forward 5′-TTCTTTCGACGTCTCTTGTGCG-3′; reverse 5′-TTGATGCTACGATCTGCGTGG-3′). After sequencing, verification PCR products were transferred into a pcDNA3 vector (Invitrogen, San Diego, CA). Large-scale preparation of plasmid DNA was conducted using MegaPrep (Qiagen, Chatsworth, CA). Cardiotoxin (Sigma-Aldrich, St. Louis, MO) was injected into the anterior muscle of the tibias of 5- to 6-wk-old C57BL/6 mice (10 μM per leg). One week after injection, the mice were injected with 100 μg DNA in PBS.

RNA extraction and real-time PCR

Prostate tissue samples were stored in liquid nitrogen until RNA extraction. RNA was extracted using TriReagent (Sigma-Aldrich), according to the manufacturer’s instructions, and was reverse transcribed with M-MLV reverse transcriptase (Promega, Madison, WI) using random primers (Amersham Biosciences, Piscataway, NJ). Quantitative PCR was performed with Absolute Blue SYBR-Green ROX Mix (Thermo Scientific, ABgene, Hamburg, Germany), according to the manufacturer’s instructions, with the Rotor-Gen eTM 6000 system (Corbett Research, Sydney, Australia) and its software, version 1.7. The amounts of transcripts were normalized to that of β-actin. Melting curves were determined to ensure the amplification of a single product. The primers used were: hCCl2, F′-CAGCAGATGACAAACTACCC, R′-TTGAACTCCGACACACTTCTT; hCCl3, F′-AGTTCCTCGACATCCTGCTGG; R′-CCCTTCGCTTGTTAGGAA; hCCl5, F′-ATCCCTCATTTTGCATGCCCT, R′-GCCACTGTTGATGAAATATCTCC; hCCl8, F′-ACTGAGAGTAGGATGAGGAGGAC, R′-AACCCCTGCTGCCAGTTC; hCCl10, F′-GTTGAGCTCAGAAGGTACCTCT; R′-GCTCTTGGATCTGATGACACA; hCCl12, F′-ATGGCCATCGCCGCTTACTCG; R′-GCCGGGTCAAAACCTGGAGAO; hCCl23, F′-CTCTAAATTGACACCCCC; R′-CCACCTGATATGCCAGAT.

For mouse primary tumor, we used the same basic protocol, yet the amounts of transcripts were normalized to GAPDH. The following primers were used: mouse CCL2, F′-TTAAAACCTGAGTCAAGAACCA, R′-GCACTTACCTCAGATTACGGGT; mouse GAPDH, F′-CATGTTCCTGAGATGACTCCAAC, R′-GGCTTCACCACCCATTTGTG; mouse CCL5, F′-GCTGCTTTGCAATCTCCTCC, R′-TGAGTGGACAAACACGACTGC; mouse CCL12, F′-GTCAGGCTGAACCTGAGTG; R′-TTCTAGGCGCTGGCAACAACT.

Macroptage depletion

Clodronate (dichloromethylene diphosphonate)-induced macrophage depletion was conducted according to Jordan et al. (27). The depletion of macrophages was confirmed by flow cytometry analysis on spleen cells (F4/80 Ab, BD Biosciences, San Jose, CA). The depletion was considered successful only if ≥98% of these cells were depleted.

Western blot analysis

Western blot analysis was conducted as described in (11).

Statistical analysis

The significance of differences was examined using the Student t test; p = 0.05 was considered statistically significant. Significance of Ab titer was determined using receiving operating characteristic curve analysis that is based on the binominal distribution to obtain upper and lower bounds for 95% confidence intervals surrounding estimates of test sensitivity and specificity (28).

Results

CaP patients display a significant auto-Ab titer to CCL2

Our study included 23 CaP patients, 21 individuals with benign prostate hyper trophy (BPH), and 11 control subjects (Table I) who were tested for the expression of different chemokines (immunofluorescence analyses of prostate gland sections and real-time PCR) and the development of auto-Ab to these chemokines as determined by ELISA on sera samples. In all subjects, sera were...
Patient demographics and characteristics

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<td>PSA, ng/ml [median (range)]</td>
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<td>3</td>
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<td>7</td>
<td>18</td>
</tr>
<tr>
<td>≥8</td>
<td>2</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>Age, y [median (range)]</td>
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The basic clinical information regarding CaP patients who participated in the study is summarized. It complements the data presented in Fig. 1. In all CaP patients included in Table I and Fig. 1, CCL2 titers and PSA levels were obtained before treatment. All patients except for one (metastatic patient with a PSA level of 3400) are patients with clinically localized disease (stages T1a, T1b, and T2).

taken before entering any therapeutic protocols (i.e., radical prostatectomy, hormone therapy, or radiation).

We first subjected human malignant primary tissues from five different patients with Gleason scores varying from 4–7 to immunofluorescence analyses for the expression of CCL2, CCL3, CXCL8, CXCL10, and CXCL12, and compared them with reciprocal samples from five BPH subjects (five sections from each subject). Fig. 1A displays representative sections subjected to comparative staining of these chemokines. Apparently, CCL2 dominates the chemokine expression within the malignant tissue (Fig. 1Aa compared with 1Ac, 1Ad, 1Ag, and 1Ai). Notably, sections from BPH subjects displayed relatively lower expression of CCL2 compared with malignant tissues (Fig. 1Ab compared with 1Ae). Computerized analyses of all sections (20 × 5 from either CaP or BPH subjects) showed significantly higher fluorescence intensity (as determined in pixels per field) of CCL2 staining in sections from CaP subjects compared with each of the other chemokines (Fig. 1B) (4.15 ± 0.9 versus 0.09 ± 0.005, 0.21 ± 0.06, 1.55 ± 0.4, 2.17 ± 0.6, p < 0.01, for the comparison of CCL2 staining to CCL3, CXCL10, CXCL8, and CXCL12 in CaP subjects, respectively) and also in comparison with CCL2 staining in BPH (Fig. 1B) (4.1 ± 0.9 versus 0.53 ± 0.07, p < 0.01). Fig. 1B also shows that the fluorescence intensity of CCL2 in CaP is ∼10-fold higher than in BPH. Within the BPH group, however, the expression of this chemokine was significantly higher than CCL3, CXCL10, and CXCL12 (Fig. 1B, p < 0.01).

A similar pattern of results was obtained when determining the transcription of each chemokine in tissue samples of CaP subjects (calibrated to β-actin) (Fig. 1C) (8.3 ± 1.1 versus 1.2 ± 0.4, 3.9 ± 0.5, 0.5 ± 0.05, 4 ± 0.9, 4.7 ± 0.8; p < 0.01, for the comparison of CCL2 transcription to the transcription of CCL3, CCL5, CXCL10, CXCL8, and CXCL12 in CaP subjects, respectively) and also for the...
comparison of CCL2 transcription in CaP subjects compared with BPH subjects (Fig. 1C) (8.3 ± 1.1 versus 2.1 ± 0.6, p < 0.01).

Then, sera from all 23 CaP patients, 21 individuals with BPH, and 11 control subjects were tested for the presence of auto-Abs to CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL8 (IL-8), CXCL10 (IP-10), and CXCL12 (SDF-1α). Of these chemokines, CaP patients mounted a highly significant Ab titer exclusively to CCL2 (Fig. 1Da, log2 Ab titer of 11.85 ± 0.8). The baseline titer of anti-CCL2 Abs (log2) in healthy individuals and in BPH patients was ~6 (p < 0.001, compared with anti-CCL2) and did not differ from the one observed in response to any of the other chemokines. A complementary examination at a single sera dilution of 1:500 revealed an exclusive 3.5-fold increase in OD (450 nM) of anti-CCL2 Ab production only in sera of CaP subjects (Fig. 1Db, p < 0.0001 compared with all other groups).

Comparative analysis of CCL2-specific Ab titer developed in CaP compared with BPH and age-matched control subjects was also conducted. Results are shown as log2 Ab titer. The cutoff (log2 Ab titer >9) was determined using ROC curve analysis as described in Materials and Methods. Thus, 82% (19/23) of CaP patients and 4.7% (1/21) of BPH patients displayed a significant response to CCL2. Taken together, these results show that selective loss of tolerance to a chemokine may occur if this chemokine is predominantly expressed at a tumor site. We have previously shown in experimental models of autoimmunity, which were extended to humans, that during inflammatory autoimmunity, but not in response to bacterial adjuvants, the immune system selectively breaks down the tolerance to key inflammatory mediators that are predominantly expressed at the autoimmune site, such as TNF-α in RA patients (3), which is predominantly expressed at the inflamed joint and is a successful target for therapy (29). Taken together, these data and our current observations suggest that, within a segregated tissue undergoing a destructive process, the immune system generates an Ab-based response to inflammatory mediators that are predominantly expressed within the tissue.

CaP cells produce CCL2 and express its CCR2 receptor (30). Targeted neutralization of CCL2 is thought to be beneficial in blocking tumor development and its spread (12, 13). As CaP patients do display an apparent anti-CCL2 Ab titer, it is an opened question whether these Abs participate in the natural regulation of the disease. The minimal requirement for such a possible beneficial involvement is the ability to neutralize CCL2. To determine this possibility, we purified sera of the previously described 23 CaP patients on a CNBr-CCL2 chemokine purification column and tested the purified Abs for their ability to inhibit CCL2-induced migration of the human CaP cell line PC-3. Fig. 1E shows that these Abs neutralized and significantly inhibited the CCL2-induced migration of this line (p < 0.001). These observations motivated us to further explore the role of anti-auto–Abs to CCL2 and their amplification as a potential therapeutic intervention.

Elicitation of auto-Ab production of CCL2 in immunocompetent mice is dependent on cancer cell tumorigenicity

TRAMP, developed by Dr. N. Greenberg, is a spontaneously developed disease in transgenic mice (C57BL/6 background) (24). Three CaP cell lines, termed C1, C2, and C3, were generated from these mice by Dr. Greenberg (24). Of these lines, C1 very effectively forms tumors in immunocompetent mice, whereas C3 lacks this ability (31). Before administering C1 and C3 lines, we verified that both produce CCL2 in cultured media (ELISA) and express its CCR2 receptor (FACS analysis, rabbit anti-mouse CCR2 [E68], Novus Biologicals, Littleton, CO). Both lines were administered to immunocompetent C57BL/6 mice. In accordance with Foster et al. (31), only C1 successfully formed an established primary tumor with a time-dependent increase in tumor volume (Fig. 2A), with apparent tumor angiogenesis, as verified by histological evaluation (not shown). Both lines produced (in vitro) comparable levels of CCL2 (not shown). Yet, real-time PCR analysis conducted on primary tumor sections isolated on day 25 from mice administered with the C1 line (Fig. 1A) showed a marked increase in the transcription of CCL2 (p < 0.0001) compared with those injected with the C3 line (Fig. 2B). Moreover, mice developing
C1-induced tumor elevation in the transcription of other chemokines, including CCL5 and CXCL12, displayed only a minor increase compared with CCL2 ($p < 0.001$). Subsequently, only C1 that successfully formed an established primary tumor also initiated the development of specific auto-Ab's to CCL2, but not CCL3, CCL5, CXCL10, or CXCL12, as presented by Fig. 2C (25 d after tumor implementation, log$_2$Ab titer of 11 ± 0.5 versus 6 ± 0.5 and 7 ± 1.66 in control groups injected with C3 or PBS, $p < 0.001$). This CCL2-specific high Ab titer continued to persist along tumor development and progression (Fig. 2D) in the vast majority of detected mice (16/18 in three independent experiments). We then purified these Abs (affinity purification), and determined their ability to inhibit the migratory properties of RAW cells in response to CCL2. Fig. 2E shows that these CCL2-specific Abs could markedly ($p < 0.0001$) inhibit the migration of these cells in a trans-well system in a similar manner to our control anti-CCL2 mAb (R&D Systems). Both Abs could not inhibit CCL5 induced migration of RAW cells (not shown).

Taken together, these results show that in humans and in a murine setting, the development of a tumor may lead to selective breakdown of tolerance that is dependent on the tumorigenicity of the transformed malignant cells.

**CCL2-encoding DNA vaccine amplifies the production of specific auto-Ab's against CCL2 and suppresses tumor development**

Because of the relevance to humans, we determined whether targeted DNA plasmid encoding CCL2 could enhance Ab production to its gene product in mice displaying anti-CCL2 Ab titer and, if so, whether this amplification affects the dynamics of tumor development. Fig. 3 summarizes the results of one of three different experiments with similar results, in which groups of six mice were injected with the $7 \times 10^6$ C1 cell line and 25 d later, when the primary tumor was established (verified also histologically in representative mice). These mice were injected every 5 d with CCL2-encoding DNA plasmid, β-actin–encoding DNA plasmid,
or PBS. Fig. 3A shows that as early as 10 d after the first plasmid DNA injection, those mice injected with CCL2-encoding DNA developed an accelerated Ab titer against CCL2 (Fig. 3A, day 35, log2 Ab titer of 17.5 ± 1.6, 9.4 ± 1.1 and 10.8 ± 0.7, respectively), but not against each of the other chemokines shown in Fig. 2C (not shown). This titer continued to persist during the progression of disease (Fig. 3A). An observer blind to the experimental protocol monitored the progression of the primary tumor.

Fig. 3B summarizes the results of one of three experiments with similar data, showing that only the administration of CCL2-encoding DNA plasmid significantly slowed tumor development (e.g., on day 45, a tumor volume of 70 ± 10 mm3 versus 410 ± 36 mm3 and 410 ± 30 mm3; p < 0.001). We also determined the level of CCL2 in sera (R&D Systems, ELISA kit) of mice subjected to CCL2-encoding DNA vaccine, compared with control mice developing C1 tumor (day 25). Our results show a 2–3-fold increase in sera levels of CCL2 in DNA vaccinated mice. These results are comparable to those we have recently reported, in which CCL2 was neutralized (in vivo) by a soluble receptor (23).

On day 70, all mice were euthanized and subjected to immunofluorescence and immunohistochemical analyses. As shown in Fig. 3C, repeated injection of DNA vaccine to CCL2 led to a marked reduction in CD31 staining (for vessels) (Fig. 3Ca versus 3Cb and 3Cc) as well as F4/80+ macrophages (Fig. 3Cd versus 3Ce and 3Cf), in accordance with decreased VEGF expression at the tumor site (Fig. 3Cg versus 3Ch and 3Ci).

CCL2-specific Abs isolated from protected donors transfer the beneficial effect of plasmid DNA therapy

The association between the acceleration of CCL2-specific Ab titer after the administration of CCL2-encoding DNA plasmid and tumor regression may suggest that these Abs suppress tumor development by neutralizing CCL2. In attempting to evaluate this hypothesis, we purified these Abs from protected donors (Fig. 3, day 70) and, after CNBr-Ag purification, we determined their in vitro neutralizing properties and then their competence to suppress the development of C1-induced primary tumor in immunocompetent mice (Fig. 4). CCL2 is a chemoattractant for the tumor cells and for tumor-associated macrophages, which are likely to facilitate tumor development. We first detected the ability of our Abs to affect the CCL2-induced migration of a commercially available macrophage cell line, RAW. As shown in Fig. 4A, anti-CCL2 Abs purified from protected donors, but not from those mice injected with β-actin–encoding DNA plasmid (only IgG purification) could markedly (p < 0.001) suppress CCL2-induced migration of RAW cells similarly to commerciality available Abs against CCL2. Subsequently, these Abs, but not those purified from mice injected with the β-actin–encoding plasmid, inhibited the CCL2-induced migration of the tumor C1 cells (Fig. 4B, p < 0.0001). Before their in vivo administration, we examined the ability of these Abs to inhibit the in vitro proliferation of C1 cells (Fig. 4C). Thus, purified anti-CCL2 Ab from donors with reduced tumor progression inhibited macrophage and tumor cell migration and also tumor growth (proliferation). What is the mechanistic basis by which CCL2 affects these functions of C1 cells? Previous studies conducted on the human PC3 line showed that CCL2 activates the PI3K/AKT pathway resulting in the phosphorylation of p70-S6 kinase (a downstream target of AKT) and induced actin rearrangement, resulting in a dynamic morphologic change indicative of micropike formation (11). Very recently, Roca et al. (32) showed that CCL2 maintains the survival of these cells via PI3K/AKT-dependent pathway. The current study shows that CCL2-induced signaling on C1 cells also includes the same pathway (Fig. 4D), which is CCL2-induced AKT phosphorylation that could be inhibited by Wortmannin: a fungal metabolite that specifically inhibits PI3K, MAPK, and myosin light-chain kinase (33), and P70 S6k phosphorylation that could be inhibited by LY294002: a specific inhibitor of PI3K, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (34).

Finally, we determined the ability of these Abs to suppress C1 cell-induced tumor development. Fig. 4E shows that repeated administration (every 5 d, 200 μg/mouse, i.v.), beginning on day 20 when tumor size was 21.6 ± 5.5 mm3, slowed tumor development (e.g., day 60 tumor volume of 170 ± 26 mm3 versus 600 ± 52 mm3 and 680 ± 71 mm3, p < 0.001). These results strongly suggest that targeted DNA plasmids encoding CCL2 amplify beneficial anti-CCL2 auto-Ab production during tumor development and, by so doing, suppress its progression.

The association between targeted neutralization of CCL2 (Figs. 3, 4), inhibition of tumor associated macrophages recruitment at the tumor site (Fig. 3C), and inhibition of tumor development may suggest that targeted neutralization of CCL2 affect tumor development, in part, by inhibiting macrophage recruitment at the tumor site. To further investigate this hypothesis, we have conducted an additional set of experiments in which three groups of mice were administered with 7 × 107 C1 cells and, 7 d later, subjected to either neutralization of CCL2 by a plasmid DNA vaccine encoding this chemokine or to a depletion of macrophages by repeated injections of clodronate liposomes in a 4-d interval (Roche Diagnostics Mannheim, Germany). Effectiveness of macrophage depletion was determined using additional mice that were treated with clodronate liposomes and sacrificed on days 8 and 12. Macrophage depletion was considered successful only if ≥98% were depleted (using a flow cytometry analysis). Fig. 5A shows that although mice treated with PBS (C1 group) and those administrated with an empty liposome continued to develop a progressive primary tumor (day 55, mean tumor volume of 490 ± 50 mm3 and 530 ± 55 mm3), those treated with either CCL2-encoding DNA plasmid or clodronate liposomes displayed a markedly reduced tumor development (170 ± 20 mm3 and 230 ± 25 mm3, respectively, p < 0.001 in comparison with each control group). Complementary immunostaining (F4/80) of primary tumor sections of each group (Fig. 5B, day 55) showed a marked decrease in the accumulation of tumor-associated macrophages in mice subjected to either CCL2-encoding plasmid DNA, or macrophage depletion (Fig. 5Be and d compared with 5Bc and 5Bb). Taken together, these results suggest that therapy with plasmid DNA encoding CCL2 leads to increased CCL2-specific Ab production, which inhibits the recruitment of tumor-associated macrophages at the tumor site, and that these macrophages are essential to support tumor development.

Discussion

We have previously shown in experiments that were initiated in experimental models and extended to humans that, during inflammatory autoimmune diseases, the immune system develops a neutralizing auto-Ab–based response to a very limited number of inflammatory mediators (3). It is tempting to assume that this response is initiated to restrain the harmful consequences of these diseases. Nevertheless, it is clear that decisions taken by the immune system are based on simplistic rules of interactions, rather than rational thinking. Our working hypothesis has been that this selective breakdown of immunological tolerance is due to a predominant expression of an inflammatory mediator at an immune-restricted site undergoing a destructive process. If so, such conditions also characterize cancer diseases, in which inflammatory mediators are highly expressed at the primary tumor site, which undergoes an immune-restricted destructive process. At the tumor site CCL2 is produced by the cancer cells, invading macrophages, and by the endothelium, all of which express its CCR2 receptor.
We show in this study that targeted neutralization of CCL2 inhibited macrophage (F4/80+) cell accumulation at the tumor site in association with decreased angiogenesis (determined by CD31 staining) and decreased VEGF could be recorded (Fig. 3C), which may explain, in part, the inhibition in tumor growth. Nevertheless, we do not have direct proof for the working hypothesis that the blockade of CCL2 directly inhibits macrophage accumulation at the tumor site. We also do not know whether

**FIGURE 4.** CCL2-specific auto-Abs produced in DNA-vaccinated mice transferred the beneficial effect of the vaccine. *A*, Inhibition of CCL2-induced chemotaxis of RAW cells by purified Abs compared with commercially available anti-CCL2 neutralizing Ab. The ability of CCL2-specific auto-Abs (50 μg/ml CNBr-CCL2–purified Abs) from DNA-vaccinated mice to inhibit CCL2-induced migration compared with those purified from mice injected with β-actin–encoding DNA or commercially available anti-CCL2 neutralizing Ab compared with CCL5-induced migration. Results are shown as the mean chemotaxis index of triplicates ± SE. *B*, CNBr-CCL2–purified Abs inhibits CCL2-induced migration of TRAMP-C1 cells. The ability of CCL2-specific auto-Abs (50 μg/ml CNBr-CCL2–purified Abs) from DNA-vaccinated mice to inhibit CCL2-induced migration was examined in a CytoSelect cell migration assay (8-μm pore size) compared with those purified from mice injected with β-actin–encoding DNA or commercially available anti-CCL2 neutralizing Ab. Results are shown as mean triplicates ± SE. *C*, The ability of CCL2-specific auto-Abs developed in mice injected with CCL2-encoding DNA plasmid to inhibit the proliferative response of C1 was compared with cultures supplemented with PBS (medium), with β-actin–encoding DNA plasmid, or with the addition of commercially available anti-CCL2 Ab. Results are shown as mean [3H]thymidine uptake of triplicates ± SE. *D*, The ability of CCL2 to induce the phosphorylation of AKT and P70-S6k in C1 cells, and of Wortmannin and LY294002 to inhibit these phosphorylations. *E*, Three groups of six C57BL/6 mice were injected with 7 × 10^6 C1 cells/mouse s.c. and treated every 5 d, starting day 20, with PBS, 200 μg/ml purified anti-CCL2 Ab, or 200 μg/ml purified anti-β-actin Ab. Mice were monitored every 5 d for the development of the primary tumor by an observer blind to the experimental protocol. Results are shown as mean ± SE of six mice per group.
neutralizing Abs against CCL2 that are likely to participate in the
regulatory network of the immune system. Our working hypothesis is that under these
conditions at first auto-reactive CD4+ T cells are activated in response
to this chemokine. Then, these cells further activate chemokine-specific B cells. This hypothesis has still to be proved.

Fig. 1 also shows that the transcription of several chemokines including CCL2, CCL5, CXCL12, and CXCL8 is selectively elevated in CaP compared with BPH. At the tumor site, these chemokines are likely to be produced by various types of cells, in particular the cancer cells, tumor-associated macrophages, and other bone marrow-derived cells that preferentially accumulate at the tumor site during malignancy (35). This may explain the accumulation in chemokine transcription and expression during malignancy and not BPH.

Production of auto-Abs to two proinflammatory chemokines CCL2 and CXCL8 after i.v. administration of LPS was previously reported (36). LPS administration leads to a rapid elevation in chemokine level in the sera, which is likely to assist this breakdown of tolerance.

Over the last 10 y, we have shown in experimental models of several inflammatory autoimmune diseases that targeted DNA plasmids encoding inflammatory cytokines/chemokines (1, 4–6, 8, 37–39). Throughout these studies we noticed that these vaccines, when injected after induction of disease, act very rapidly, because they amplify a pre-existing response initiated by the autoimmune process (1–3, 8, 39). Nevertheless, the mechanistic basis of how the immune system generates a selective breakdown of tolerance under an autoimmune condition, but not after adjuvant-induced local inflammatory response (3), or how it restricts the response to a very limited number of mediators, remains elusive. One must understand that this system is incapable of making its own rational decisions regarding what could be beneficial for the regulation of an autoimmune process or malignancy.

We have previously shown that in RA, the immune system of these patients selectively mounts an auto-Ab response to TNF-α (3) that is predominantly expressed at the autoimmune site (40). Very recently, we showed that during T1DM, diabetic subjects display a selective auto-Ab response to CCL3 (9). We think that in humans this chemokine is predominantly expressed at the autoimmune site, although the inaccessibility of human pancreas samples from these patients precludes indepth examination of this assumption. A common denominator of tumor development and autoimmunity is that both include a “pathogen-free” destructive process, in which inflammatory mediators, including chemokines are highly expressed at an immune-restricted site. Similar conditions occur in solid cancers. This motivated us to expand our study to cancer diseases, where the accessibility of primary tumor sections from human subjects is more accessible. The current study, using human cancer primary tissue samples, together with our early publication using RA samples, (3) imply that, regardless of the nature of disease, the predominant expression of an inflammatory mediator at an immune-restricted site, under destructive conditions, would provide appropriate conditions for selective Ab-based loss of immunological tolerance.

Even though CaP patients do display an apparent anti-CCL2 Ab titer (Fig. 1A) that should protect them, they develop a progressive disease. Why so? It is likely that as neutralizing Abs, they induce a beneficial effect, but their low titer is perhaps sufficient to restrain but not fully suppress its development and progression.

The role of CCL2 in cancer, particularly CaP and other androgen-dependent cancer diseases, could be viewed from two complementary perspectives: its direct effect on recruitment and growth of

FIGURE 5. Depletion of macrophages suppresses the development of the primary tumor. A, Three groups of mice were administered with 7 × 10⁶ C1 cells, and 7 d later subjected to either neutralization of CCL2 by a plasmid DNA vaccine encoding this chemokine (closed circles), or to depletion of macrophages by repeated injections of clodronate liposomes, in a 4-d interval (closed square). Control mice were treated with PBS (open circles) or an empty liposome (closed triangles). Mice were monitored every 5 d for the development of the primary tumor by an observer blind to the experimental protocol. Results are shown as mean ± SE of five mice per group. B, Comparative immunostaining of macrophages (F4/80) at the primary tumor (day 55) of the primary tumor of mice injected with either: PBS (a), empty liposomes (b), plasmid-encoding CCL2 (c), or clodronate liposomes (d). Each section represents 18 different sections of 6 mice per group.

reduced VEGF and possibly other growth factors at the tumor sites of these mice is due to a direct effect of macrophages that are likely to produce them at the tumor site, or due to their interaction with other cells there. Fig. 5 shows that direct depletion of macrophages also inhibited tumor growth, partially supporting the hypothesis that these cells support tumor growth either directly or because of their interaction with other cells at the tumor site.

We show that of the various chemokines associated with CaP, CCL2 is predominantly expressed at the primary tumor site of these patients, and that they develop a selective neutralizing auto-Ab titer in response to CCL2. We then used an immunocompetent model of the disease to show that targeted amplification of this response led to tumor regression, thus demonstrating a practical way of combating the disease.

Lu et al. (12) showed in 2006 that CCL2 expression correlates with pathology in human CaP. These results are in accordance with our current study (Fig. 1D, 1E). Using a xenograft model of CaP in SCID mice, the same group showed in 2007 that Ab-based blockade of CCL2 induces tumor regression in vivo (13). Subsequently, very recently, we published similar observations using an Ig-CCR2–based fusion protein that preferentially binds and neutralizes CCL2 (23). These results, together with those of the current manuscript (Fig. 4), suggest that CCL2 is likely to be an important target for therapy for CaP. Selective breakdown of tolerance to this chemokine in humans (Fig. 1) may suggest an applicable way of therapy for CaP patients. The current study shows that patients who have CaP display an apparent auto-Ab titer of neutralizing Abs against CCL2 that are likely to participate in the regulation of disease in a beneficial manner. These Abs could be used either for diagnosis of the malignant stage of disease, or as a target for beneficial amplification. The mechanistic basis of how the immune system selectively breaks down tolerance to chemokines that are preferentially expressed at the tumor site is far from being understood. Our working hypothesis is that under these conditions at first auto-reactive CD4+ T cells are activated in response to this chemokine. Then, these cells further activate chemokine-specific B cells. This hypothesis has still to be proved.
malignant cells (12, 13), and its role in recruiting tumor-associated macrophages to assist tumor invasion (41–45). Focusing on the role of the CCL2–CCR2 interaction in macrophage recruitment at the tumor site, we show in this study that, indeed, targeted neutralization of CCL2 inhibits the migration of tumor-associated macrophages to the tumor site, and at the same time, leads to markedly decreased expression of the major angiogenic factor VEGF, which directs tumor implementation (46).

One possible ramification of our study is its potential extrapolation in humans. This strategy holds advantages and drawbacks compared with Ab or soluble receptor-based therapies. The major advantages are that it interferes, by amplification, a natural process of targeted neutralization. Such amplification could lead to auto-development of improved therapy because of affinity maturation of the chemokine-specific Abs, although this hypothesis has yet to be proven. The two major drawbacks of DNA-based vaccines, aimed at amplifying beneficial immunity are as follows: 1) Once amplified, such a response is regulated, independently, by the dynamics of the disease. For example, TNF-α-encoding DNA vaccines could be used as a practical way of combating RA. However, it might become problematic if the subject undergoes pregnancy. Because CaP is a terminal disease in men, and because mice lacking CCL2 display minimal side effects, this drawback is limited. 2) Previous studies, by several groups, including ours, showed that in the absence of anti-self–Abs, DNA vaccines might generate an opposing effect. That is, without Ab-based neutralization the gene product of the vaccines would be functional and provoke an effect opposing the one desired by the vaccine (47–49). This strategy has been successfully implemented for therapeutic target expression of gene products at autoimmunites sites (47–49). Thus, potentially, it is possible that in some patients who have CaP, CCL2-encoding vaccine would induce an opposing effect. A simple way to avoid this difficulty could be to use a plasmid DNA encoding an N-terminal truncated CCL2, in which the gene product is inactive (50).

The reason for selecting the C1-induced disease as a model to investigate the validity of our findings is that in this model, as with the human disease, CCL2 dominates the chemokine expression at the tumor site. C1 very effectively forms tumors in immunocompetent mice, whereas C3 lacks this ability as shown by others (31) and verified in this study (Fig. 1A). Subsequently, of these lines only C1 led to an increased transcription of CCL2 at the tumor site (Fig. 2B). This could result not only from direct production of this chemokine by the tumor cells, but also from an upregulation of CCL2 transcription by macrophages and by the stroma cells that support the tumor (51). Nevertheless, the consequence of this dominant transcription is the selective loss of tolerance to CCL2 leading to the development of CCL2-specific auto-Abs (Fig. 2C) that continue to persist along the development of the tumor (Fig. 2D), and as neutralizing Abs (Fig. 2E) possibly participate in its regulation. By showing that targeted amplification of their function suppress tumor development, we suggest an applicable way of therapy that could also be explored in humans.

In an attempt to further validate our findings, we investigated selectivity of tolerance breakdown in TRAMP mice due to overexpression of SV40 develop CaP (24). Surprisingly, we found that, even though the C1 line is derived from TRAMP mice (24), in these mice the expression of the CCR5 ligands CCL3 and CCL5 at the primary tumor overrides CCL2 that is also apparently expressed, although to a lower relative level (not shown). Subsequently, these mice generate selective auto-Ab titers to these chemokines over CCL2. These results further support our working hypothesis that selective breakdown of immunological tolerance in humans and mice is directed by dominantly expressed inflammatory mediators at an immune-restricted site undergoing a destructive process. CCR5 ligands have been recently suggested to serve as growth/survival factors for androgen-dependent cancer diseases, including CaP (52, 53). In future studies, we intend to use this model and the DNA vaccination technology to further investigate their role in CaP.

The other applicative outcome of our study also refers to the diagnosis of the malignant stage of disease. The current biomarker commonly used for the diagnosis of CaP is sera levels of prostate-specific Ags (PSAs) that are elevated in accordance with hyper-trophy of the prostate gland and cannot predict transition to malignancy (54, 55). Another limitation of measuring PSAs as a major screening tool for the diagnosis of CaP is that significant portions of malignant subjects (~15%) have low levels of PSAs with low velocity (56). It remains to be examined how disease-specific is the production of CCL2-specific Abs. Yet, it could serve as an important complementary or even alternative biomarker that would not only identify the transformation from benign hyper-trophy to malignancy, but also identify low levels of PSAs in subjects with developing CaP.

There is an open-ended question about the in-depth mechanistic basis of immunological tolerance breakdown under the conditions in which inflammatory mediators are predominantly expressed at a restricted tumor or autoimmune site undergoing intensive destruction. We think that natural adjuvants relapsed from destructed dying cells provide the appropriate environment for tolerance breakdown (57). Future studies will elaborate this hypothesis.

Finally, it should be noted that effective neutralization of CCL2 slows tumor development, but does not eradicate it. Thus, it is likely that other factors are involved in containing the tumor. This suggests that an effective therapy for CaP should combine inhibition of a key mediator that supports tumor development, such as CCL2, together with other ways of therapy, as was recently demonstrated in a study that used anti-CCL2 and docetaxel as an effective combined therapy (13).

Disclosures
N.K. and G.W. hold pending patents on the therapy of autoimmunity and cancer using CCL2-encoding DNA vaccines, and on using anti-CCL2 Abs for the diagnosis of prostate cancer.

References


Corrections


The seventh author’s name was omitted from the article. In addition, the third author’s name was published incorrectly. The corrected author and affiliation lines are shown below.

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