The CCL3 Family of Chemokines and Innate Immunity Cooperate In Vivo in the Eradication of an Established Lymphoma Xenograft by Rituximab[1]

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The therapeutic mAb rituximab induced the expression of the CCL3 and CCL4 chemokines in the human lymphoma line BJAB following binding to the CD20 Ag. Induction of CCL3/4 in vitro was specific, was observed in several cell lines and freshly isolated lymphoma samples and also took place at the protein level in vitro and in vivo. To investigate the role of these β-chemokines in the mechanism of action of rituximab, we synthesized a N-terminally truncated CCL3 molecule CCL3(11–70), which had antagonist activity on chemotaxis mediated by either CCL3 or BJAB supernatant. We also set up an established s.c. BJAB tumor model in athymic mice. Rituximab, given weekly after tumors had reached 250 mm^2, led to complete disappearance of the lymphoma within 2–3 wk. Treatment of mice with cobra venom factor showed that complement was required for rituximab therapeutic activity. Treatment of BJAB tumor bearing mice every 2 days with the CCL3(11–70) antagonist, starting 1 wk before rituximab treatment, had no effect on tumor growth by itself, but completely inhibited the therapeutic activity of the Ab. To determine whether CCL3 acts through recruitment/activation of immune cells, we specifically depleted NK cells, polymorphonuclear cells, and macrophages using mAbs, clodronate treatment, or Rag2$^{-/-}$c$\gamma^{-/-}$ mice. The data demonstrated that these different cell populations are involved in BJAB tumor eradication. We propose that rituximab rapidly activates complement and induces β-chemokines in vivo, which in turn activate the innate immunity network required for efficient eradication of the bulky BJAB tumor. *The Journal of Immunology,* **2007, 178: 6616–6623.**

The chimeric unconjugated monoclonal IgG1 Ab rituximab (Mabthera, Rituxan) is approved for the treatment of low- or high-grade non-Hodgkin's B lymphoma and is shown in therapeutic activity in other B cell neoplasms such as B cell lymphocytic leukemia and diffuse large B cell lymphoma (1–5). Although rituximab has shown clear clinical activity in different non-Hodgkin's B lymphoma subtypes in phase III trials, identifying the factors that determine the clinical response and the efficacy of the Ab is still a major challenge. The mechanism of action of rituximab in vivo is thought to depend mostly upon immune-mediated mechanisms, but the relative contribution of different effector mechanisms is a matter of large debate (6). Different animal models of lymphoma have been described that have emphasized a role for either Ab-dependent cytotoxicity by NK cells and neutrophils (7, 8) or for phagocytosis mediated by the monocyte network (9, 10) or by complement activation (11–13). These mechanisms, however, are likely to be linked to each other. For example the complement cascade may cause direct target cell lysis by the membrane attack complex as well as induce recruitment and activation of different immune effector cells after activation and release of potent anaphylatoxins like C5a and C3a (14, 15). Finally, the role of different mechanisms is likely to vary in different tumor types or localizations (10).

Rituximab binding to the CD20 molecule also activates restricted intracellular signaling pathways. Recently we have investigated these direct effects of rituximab on lymphoma cells using a gene chip screening approach (16) and found several genes induced by the Ab in different lymphoma cells. Two of the genes of biological interest were CCL3 (Mip1-α) and CCL4 (Mip1-β), which were up-regulated in BJAB lymphoma cells in vitro after stimulation with rituximab. CCL3 and CCL4 belong to the family of β (CC) chemokines expressed generally in a coordinated fashion by a number of cell types, including activated B cells, monocytes, mast cells, fibroblasts, and epithelial cells (17). They are highly conserved between mouse and human models (18) and both signal through the CCR1 and CCR5 receptors. These receptors are also shared by other β-chemokine family members such as CCL5 (RANTES). CCL3 and CCL4 induce the infiltration of neutrophils, macrophages, NK cells, and T cells in tumors. CCL3 has been implicated in antitumor activity in several mouse models of solid tumors (19–23). In addition CCL3 has the capacity to activate certain cell types (20, 24), to affect proliferation of hemopoietic cells (25, 26), and has osteoclastic properties in vitro or in vivo

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(27). Furthermore, CCL3 plays an important role in different models of inflammation in vivo (28–30). Given the important biological activities of CCL3/4, we have investigated whether rituximab induces these proteins in vitro and in vivo and whether these chemokines played a role in the therapeutic activity of rituximab in a xenograft model of lymphoma.

Materials and Methods

Cells and Abs

The human Burkitt's lymphoma cell line BJAB (31) was cultured in RPMI 1640 medium (Seromed) supplemented with 10% FCS (HyClone Laboratories), 2 mM glutamine (Invitrogen Life Technologies), and 100 IU/ml penicillin/streptomycin. Bone marrow mononuclear cells from two Burkitt's lymphoma patients (pt1 and pt2) and one ALL-L3 patient (pt3) were separated on a Ficoll-Hypaque density gradient. Neoplastic cells in these samples were 85–99%. Rituximab was a gift of Roche Italia. Alemtuzumab (Campath-1H) was donated by Schering-Plough (Kenilworth, NJ). The humanized anti-CD25 Ab daclizumab (Zenapax; Roche) was provided by Dr. A. Rambaldi (Ospedali Riuniti, Bergamo, Italy). The purified Abs HD239 (CD22), HD50 (CD23), and HD28 (CD37) were a gift from Dr. G. Moldenhauer (DKFZ, Heidelberg, Germany) and stained BJAB cells positively. Purified rituximab $F(ab')_2$ was a gift from Prof. E. S. Vitetta (University of Texas, Dallas, TX).

Real-time PCR

Total RNA was extracted in guanidinium isocyanate and purified by standard cesium chloride gradient centrifugation. CCL3 and CCL4 gene expression was quantified in reverse transcription RNA by real-time PCR using the SYBR Green Master Mix (Applied Biosystems) and 300 nM each of oligonucleotide and normalization against β-actin, as previously described (16). The oligonucleotides were as follows: CCL3 5′-ACTACTT TGAGACGAGCAGCCAG and 5′-GCCGGCTTCGCTTGGT; CCL4 5′-CTCTCAGCACCAATGGGCTC and 5′-GTAAGAAAAGCAGCAGGC GG; and β-actin 5′-CCCAAGGCCAACCGCGAGAAGAT and 5′-GT CCCGGCCAGCCAGGTCCAG.

Tumor model

The therapeutic effect of rituximab was tested in all cases on established tumor and was analyzed as follows. Five-week-old male CD-1 nu/nuBR mice (Charles River Breeding Laboratories) were used. Groups of 3.0 Gy irradiated animals were s.c. inoculated with 10×10^6 BJAB cells in each flank, and tumor growth was measured twice weekly. When the tumor reached ~250 mm^2 (within 3–4 wk, but defined as day 0 with respect to rituximab treatment for uniformity), the mice were i.p. treated once a week with 250 μg of rituximab, control irrelevant Ab daclizumab, or saline. All results of in vivo studies of tumor growth are presented with week 0 (t = 0) corresponding to the first rituximab treatment. All procedures with animals were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

Depletion of complement in vivo

To test the role of complement, groups of mice were treated with 25 U of cobra venom factor (CVF)[3] from Quidel or PBS only on days −1, 2, 4, and 8 with respect to the first rituximab treatment in the BJAB model.

Depletion of NK cells, polymorphonuclear (PMN) cells, and macrophages in vivo

In experiments to test the role of NK and neutrophils, groups of CD-1 nu/nuBR mice were used. Mice were i.p. inoculated twice a week with the anti-NK cell Ab TM-β1 recognizing the murine IL-2R β-chain (32) and with anti-GR1 Ab RB6-8C5 specific for murine neutrophils (33), starting 2 days before rituximab inoculation, as previously described (11). In some experiments, Rag2 and cytokine receptor common γ chain double knockout mice ($Rag2^{-/-}c\gamma^{-/-}$) in a C57BL background lacking T and B lymphocytes as well as NK cells were used (Taconic Farms). These mice were s.c. inoculated with BJAB cells and treated with the standard doses of rituximab as described for athymic mice. To test the role of macrophages, groups of mice were treated i.p. with three injections of 150, 60, and 60 μl of clodronate-liposomes or PBS-liposomes on days −1, 6, and 13, respectively, relative to the first rituximab administration (34), as previously described (13). This procedure has been shown to deplete phagocytic macrophages from spleen, liver, and lymph nodes (35–38). Clodronate-liposomes and PBS-liposomes were obtained from Dr. N. van Rooijen (Vrije Univeriteit, Amsterdam, The Netherlands) and were prepared as described previously (39).

CCL3 antagonist synthesis and treatment

The CCL3 antagonist was synthesized following the sequence of aa 11–70 of human CCL3 and was named CCL3(11–70). It was synthesized by step-wise solid phase methods using tBoc protection chemistry. After hydrogen fluoride deprotection, the polypeptides were folded and purified by a minimum of two rounds reverse phase HPLC. Purity of the products was assessed by ion-exchange HPLC and mass spectrometry. The measured mass of the final product as determined by electrospray mass spectrometry was consistent with the average mass calculated from the atomic composition. The final product in PBS was stored at −80°C in aliquots for single use. For in vivo assays, groups of mice were inoculated with the standard 10×10^6 BJAB cells in each flank. After 16–20 days when tumors started to become palpable, which is 1 wk before the first rituximab treatment (t = week −1), antagonist treatment was initiated, giving 125 μg of CCL3 antagonist (aa 11–70) i.p. every 2 days for a total of eight treatments. After four antagonist treatments (t = 0), the standard dose of rituximab or control saline was i.p. administered. Antagonist treatment was maintained up to day 7 with respect to the first rituximab inoculation (t = week +1). Rituximab was given on a weekly basis for a total of three inoculations and tumor growth was recorded every 2 days.

Chemotaxis assays

$CD14^+$ monocytes were purified from PBMC from normal volunteers by positive selection using anti-CD14 microbeads (Miltenyi Biotec). Cell migration was assayed by standard chemotaxis assays using 48-well modified Boyden chamber (NeuroProbe) and 5-μm pore-size polyvinylpyrrolidine-free polycarbonate filter membranes (NeuroProbe). Different dilutions of CCL3 agonist (R&D Systems), CCL3(11–70) antagonist, BJAB supernatant, or rituximab alone were added in duplicate wells to the lower chamber, and 5×10^4 $CD14^+$ cells/well were added to the upper chamber in the presence or absence of antagonist. Chemotaxis was measured by cell count of stained filters collected after 1 h incubation at 37°C. Filters were stained with Diff-Quik solutions (Dade Behring Spa), and 5 unit fields per filter were counted at ×50 magnification using a Zeiss microscope. Migration index is defined as the mean number of migrated cells in treated samples divided by cells migrated in medium only.

Detection of CCL3 and CCL4 proteins

For detection of chemokine secretion in vitro, BJAB cells were plated in the presence or absence of 10 μg/ml rituximab. After 24 h at 37°C, supernatants were collected and analyzed for CCL3 and CCL4 content using colorimetric ELISA kits (Endogen). For detection of CCL3 induction in vivo, 10×10^6 BJAB cells were s.c. inoculated in CD-1 nu/nuBR mice as described. When the tumor reached ~200 mm^2, 250 μg of rituximab or saline was i.v. administered to different mice. After 4 h, mice were sacrificed and tumors were collected, fixed in formalin, and embedded in paraffin. CCL3 in tumor tissue was detected by standard immunohistochemistry using goat anti-human CCL3 Ab and the HRP-DAB system (R&D Systems).

Statistical analyses

Statistical analyses between different experimental groups were performed using the Student's t test. Statistical significance was set at a value of $p < 0.05$.

Results

Rituximab induces CCL3/4 mRNA and protein expression in lymphoma lines and freshly isolated samples

We have previously shown by gene chip analysis that rituximab induces CCL3 and CCL4 mRNA expression 2- to 10-fold in the BJAB as well as other B lymphoma cell lines in vitro (16). No other chemokine was found to be up- or down-regulated in the gene chip analysis (16). We further wanted to verify the specificity of CCL3/4 induction. BJAB cells were stimulated for 4 h in the presence or absence of 10 μg/ml rituximab, the humanized IgG1 anti-CD52 Ab alemtuzumab, or mouse mAbs against CD22, CD23, and CD37 molecules that are all expressed on BJAB cells

[3] Abbreviations used in this paper: CVF, cobra venom factor; PMN, polymorphonuclear.

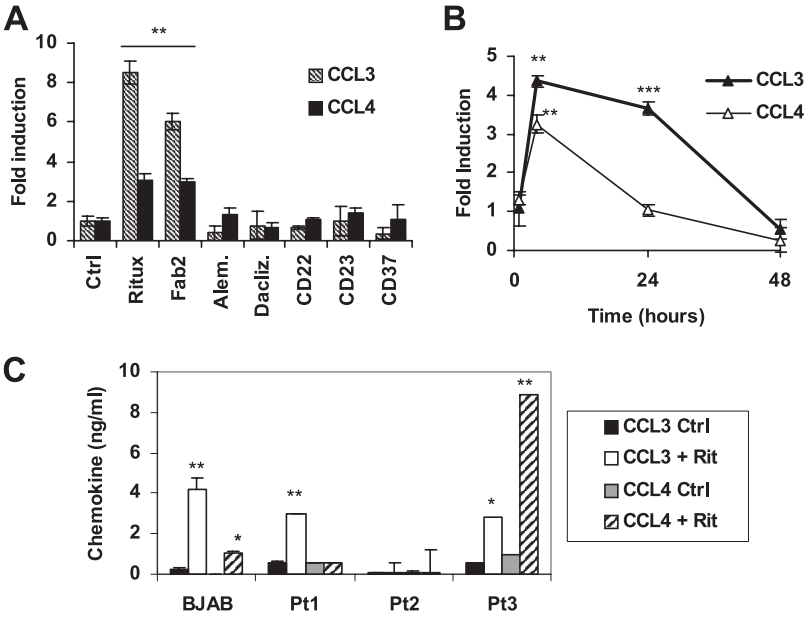

FIGURE 1. Rituximab up-regulates CCL3/4 mRNA and protein expression in vitro and in vivo. *A*, Exponentially growing BJAB cells were plated in the presence of 10 μg/ml rituximab (ritux), its F(ab′)$_2$, anti-CD52 (alem), anti-CD25 (dacliz), or murine anti-CD22, anti-CD23, or anti-CD37 Abs. Total RNA was extracted after 4 h. Fold induction over control of CCL3 (▨) and CCL4 (■) mRNA was determined by real-time PCR. The results are representative of two independent experiments. *B*, BJAB cells were plated in the presence of 10 μg/ml rituximab or control daclizumab Ab, and RNA was extracted after 1, 4, 24, or 48 h. Fold induction of the CCL3 (▲) and CCL4 (△) over control cells was determined by real-time PCR. The results are representative of two independent experiments. *C*, Cells from the BJAB cell lines from two Burkitt's lymphoma patients (pt1 and pt2) and one ALL-L3 patient (pt3) were plated in the presence of 10 μg/ml rituximab (Rit) or control daclizumab (Ctrl) Ab, and supernatants were collected at 24 h. Amounts of CCL3 and CCL4 proteins in the supernatant was determined by ELISA. Results are the mean and SD of triplicate wells. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

(data not shown). CCL3 and CCL4 mRNA expression was then analyzed by quantitative PCR. As shown in Fig. 1*A*, CCL3 and CCL4 were specifically induced by rituximab but not by any of the other mAbs, with an 8- and 3-fold increase over controls, respectively ($p < 0.001$), whereas other Abs were inactive. CCL3 and CCL4 were also induced ($p < 0.001$) by the F(ab′)$_2$ of rituximab, indicating that induction did not require the Fc portion of the Ab (Fig. 1*A*). The kinetics of CCL3 and CCL4 induction in vitro were also analyzed at 4, 24, and 48 h. Both β-chemokines showed a peak mRNA induction at 4 h that gradually decreased at 24 and 48 h (Fig. 1*B*). CCL3/4 mRNA expression was studied in five other lymphoma cells lines. Rituximab induced CCL3 and CCL4 expression in three of the five cell lines, by 2- to 30-fold and 2- to 3-fold, respectively (data not shown) (16).

We next determined whether up-regulation of the mRNA was accompanied by induction of the proteins. BJAB cells were incubated in the presence or absence of rituximab, and supernatant was collected after 24 h and analyzed for CCL3 and CCL4 by ELISA. As shown in Fig. 1*C*, rituximab induced a 20-fold increase in CCL3 and CCL4 protein secreted by BJAB cells, which reached 4 and 1 ng/ml in the supernatant, respectively. As expected the irrelevant Ab daclizumab had no effect on CCL3 protein expression (data not shown). Furthermore, no induction of CCL22 or IL-8 tested as controls was detected (data not shown). To further determine whether CCL3 and CCL4 induction also takes place in freshly isolated neoplastic B cells, cells from three patients (two patients with Burkitt's lymphoma and one ALL-L3 leukemia patient) were incubated with rituximab or control Ab, and CCL3/CCL4 was measured in the supernatant after 24 h. As shown in Fig. 1*C*, rituximab induced CCL3 in two of three patients, while CCL4 was induced in one patient.

Because the CCL3 protein was generally more highly expressed in vitro than CCL4, we investigated whether the CCL3 protein induction could also take place in vivo. For this purpose, irradiated athymic mice were s.c. inoculated with 10×10^6 BJAB cells. After the tumor had reached ~250 mm^2 (3–4 wk after inoculum), animals were treated with 250 μg of rituximab or with saline only. Animals were sacrificed and tumors excised and fixed for analysis of CCL3 protein expression by immunohistochemistry. As shown in Fig. 2*A*, increased CCL3 protein expression was detected 4 h after rituximab inoculation. Staining was specific as shown by incubation with secondary Ab only (Fig. 2*A*). Similar results were obtained at 16 h (data not shown).

We conclude that rituximab binding to CD20 specifically induces CCL3 and CCL4 mRNA and protein expression in several B lymphoma cells, and that this mechanism also takes place in vivo.

Rituximab treatment in vivo eradicates the BJAB tumor in a complement-dependent manner

To further characterize the BJAB s.c. model, we next investigated the therapeutic activity of rituximab in this context. Groups of mice were inoculated with BJAB cells and, after the tumor had developed to ~250 mm^2, a weekly 250-μg rituximab treatment was started (day 0). Tumor size was recorded weekly for 3 wk. As shown in Fig. 2*B*, rituximab treatment eradicated the tumor within about 3 wk. This effect was highly reproducible ($p < 0.0001$) and was not observed with the control irrelevant Ab daclizumab (see below and data not shown).

Because previous syngeneic models in fully immunocompetent animals (11, 13), as well as xenograft models in SCID mice (12), had suggested that complement is required for the therapeutic activity of rituximab, we next verified this hypothesis in our established BJAB s.c. model. Tumor inoculated animals were treated with CVF on days −1, 2, 4, and 8 (Fig. 2*C*, filled arrows), with respect to the first rituximab treatment. Longer treatment was not possible because CVF is antigenic. As shown in Fig. 2*C*, CVF reduced rituximab efficacy dramatically ($p < 0.0001$) during the first, second, and to some extent third Ab infusion (Fig. 2*C*, open arrows). As expected, the effect was reversible and, upon continued rituximab administration in animals previously treated with CVF, the tumor mass started to decrease significantly after the fourth rituximab administration in this group of mice (Fig. 2*C*). We conclude that rituximab is a very effective therapeutic agent in the established BJAB s.c. model and that complement is required for this activity.

N-terminally truncated CCL3(11–70) has antagonist activity

To be able to investigate the role of CCL3/4 in the mechanism of action of rituximab in vivo, we looked for an antagonist of these proteins. CCL3 and CCL4 are highly homologous chemokines that share the CCR1 and CCR5 receptors. The N-terminal amino acids of several β-chemokines are known to be important for receptor

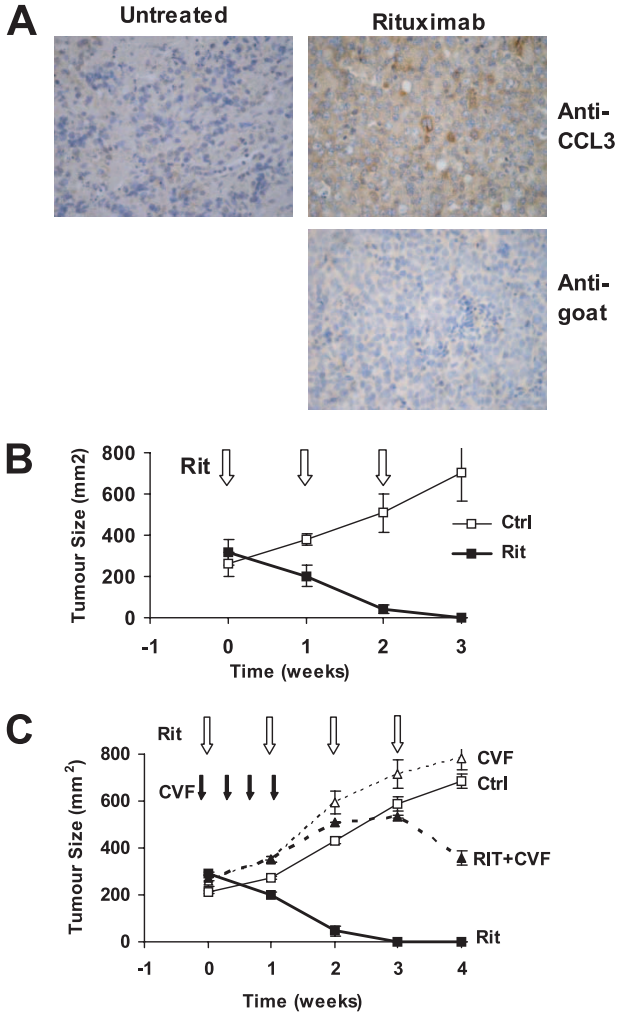

FIGURE 2. Rituximab activity in the established BJAB B lymphoma model in vivo. *A*, A total of 10×10^6 BJAB cells were s.c. inoculated in irradiated athymic mice. After the tumors had developed, the mice were treated with 250 μg of rituximab i.p., mice were sacrificed, and tumor tissue collected and fixed at 4 h. Tissue sections were stained with anti-human CCL3 Ab or secondary anti-goat Ab as control. Original magnification is $\times$20. The results represent two independent experiments. *B*, A total of 10×10^6 BJAB cells were inoculated as in *A*. After the tumors had developed to ~250 mm^2, the mice were treated weekly i.p. with either 250 μg of rituximab (Rit) (■) or saline (Ctrl) (□) once a week, and tumor growth measured once a week ($n = 5$ mice per group). *C*, Mice were inoculated as in *B*. When tumor developed, groups of mice ($n = 5$) were treated with CVF (days -1, 2, 4, and 8) (triangles) or PBS only (squares) and 1 day later with weekly injections of 250 μg of rituximab (closed symbols) or saline only (open symbols), with tumor growth recorded once a week. Arrows indicate the timing of the different treatments. Data are representative of at least two experiments.

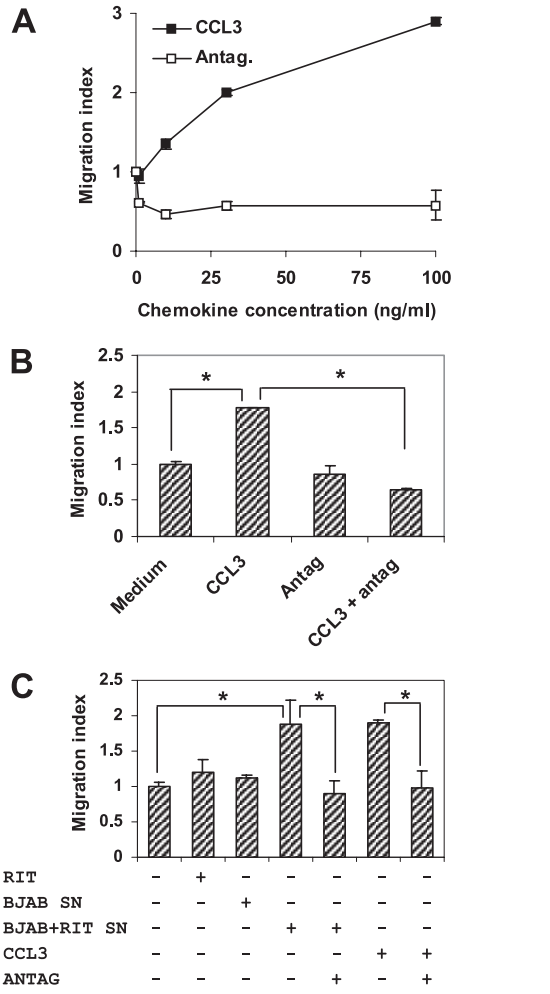

FIGURE 3. CCL3(11–70) is an antagonist of CCL3 in vitro. *A*, The migration of CD14$^+$ monocytes in response to 1–100 ng/ml CCL3 or CCL3(11–70) antagonist was measured in chemotaxic assays. Results are the mean and SD of three independent experiments. *B*, The effect of incubating the target cells with 100 ng/ml CCL3(11–70) antagonist on the migration of CD14$^+$ cells to 100 ng/ml CCL3 was determined. Results are the mean and SD of two independent experiments. *C*, BJAB cells were unstimulated or stimulated with rituximab (10 μg/ml), and supernatant (SN) collected after 24 h culture. Monocytes were then tested in chemotaxis assays against these supernatants (culture medium), rituximab alone, or CCL3 (50 ng/ml) in the presence or absence of CCL3(11–70) antagonist (50 ng/ml). $*$, $p < 0.05$.

activation, N-terminal truncations, or modifications resulting in antagonist proteins (reviewed in Ref. 17). We have therefore produced and purified a N-terminally truncated CCL3 peptide encoding aa 11–70 of the full-length protein and obtained a molecule that competed for CCL3 binding in vitro (data not shown). The antagonistic properties of the CCL3(11–70) molecule were then analyzed in chemotactic assays in vitro. Recombinant wild-type CCL3 induced migration of purified monocytes with maximal activity at 50–100 ng/ml (Fig. 3*A*). In contrast, CCL3(11–70) was inactive in chemotactic assays, when tested up to 200 ng/ml (Fig. 3*A* and data not shown). We then measured the capacity of the antagonist peptide to inhibit CCL3-mediated migration of monocytes. As shown in Fig. 3*B*, cells incubated in the presence of CCL3(11–70) failed to migrate to optimal concentrations of CCL3 (100 ng/ml; $p < 0.05$). These data demonstrate that the truncated CCL3(11–70) peptide has antagonist activity for CCL3 in vitro.

Because BJAB cells secrete CCL3 following incubation with rituximab, we also investigated whether the CCL3 antagonist was able to inhibit migration of cells induced by BJAB supernatant. As shown in Fig. 3*C*, monocytes migrated in response to BJAB supernatant collected 24 h after rituximab treatment ($p < 0.05$), but they did not migrate significantly to supernatant from either unstimulated cells or cells stimulated with rituximab alone. Furthermore, monocyte migration to stimulated BJAB supernatant was blocked by CCL3(11–70) to a similar extent as that to recombinant CCL3 ($p < 0.05$). These data suggest that rituximab binding to BJAB can induce secretion of functionally active CCL3 that can be inhibited by the antagonist.

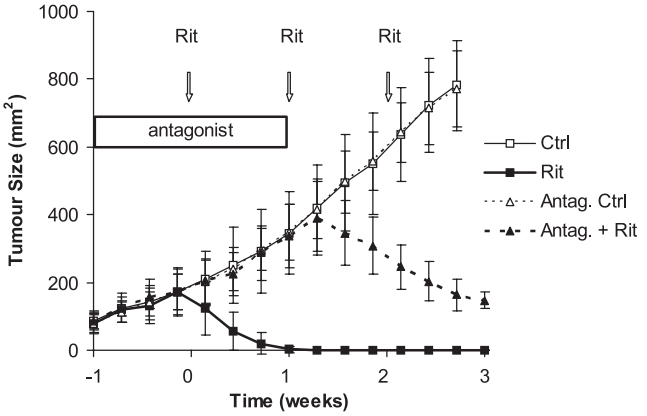

FIGURE 4. The CCL3(11–70) antagonist abolishes the therapeutic activity of rituximab in a reversible manner. A total of 10×10^6 BJAB cells were s.c. inoculated in irradiated athymic mice. After tumor became palpable, two groups ($n = 5$ mice) were repeatedly treated every 2 days with 125 μg of CCL3(11–70) antagonist (triangles) (week -1 with respect to rituximab treatment) or saline only (squares). One week later (week 0), standard treatment i.p. with 250 μg of rituximab (open symbols) or saline only (closed symbols) was initiated. The last antagonist treatment was administered at week 1 together with the second rituximab injection. A third rituximab inoculation was given on week 2. Tumor growth was measured every 2 days. The gray box (antagonist) indicates the duration of antagonist treatment and arrows represent the timing of rituximab administration. Results are representative of two independent experiments.

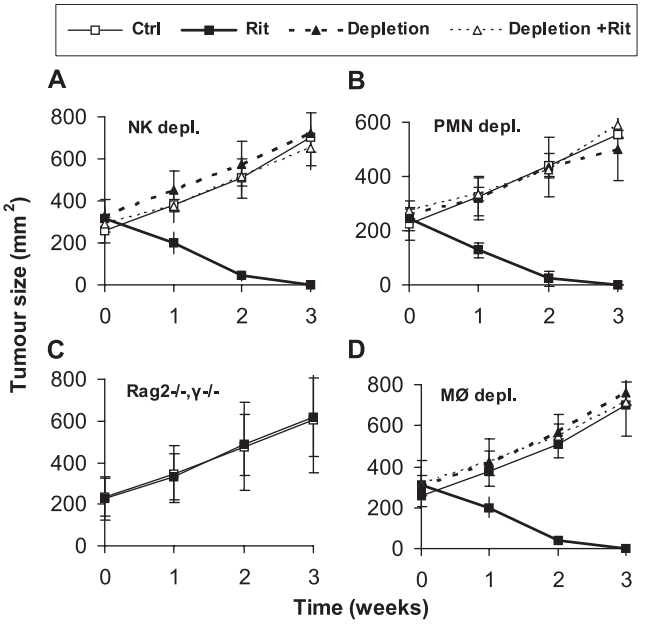

FIGURE 5. Immune cells are required for the therapeutic activity of rituximab in the BJAB lymphoma model. Athymic irradiated mice were inoculated s.c. with 10×10^6 BJAB cells. When the tumors reached ~250 mm^2, groups ($n = 5$ mice) were repeatedly treated with anti-NK Ab (*A*), anti-PMN Ab (*B*), or clodronate-liposomes (Mϕ) (*D*) (triangles) or with saline (Ctrl) (squares) followed by weekly rituximab (closed symbols) or saline only (open symbols). *C*, Rag2$^{-/-}$c$\gamma^{-/-}$ mice were s.c. inoculated with 10×10^6 BJAB and then treated with the standard rituximab weekly schedule (closed symbols) or saline only (open symbols). Tumor growth was recorded weekly. All results are representative of two experiments.

The antagonist CCL3(11–70) inhibits the therapeutic activity of rituximab in the BJAB tumor model

We next investigated the role of CCL3 in vivo in the mechanism of action of rituximab, using the BJAB tumor model and CCL3(11–70) antagonist. We first evaluated whether antagonist treatment affected tumor growth in absence of rituximab. For this purpose, 10 mice were inoculated with the standard dose of BJAB. When tumor growth started to become palpable (after 16–20 days), half the animals were i.p. treated with 125 μg of CCL3(11–70) antagonist every 2 days, and tumor growth was measured. The growth curve of the tumor was the same in the presence or absence of antagonist treatment (Fig. 4). We then determined the effect of antagonist on the therapeutic activity of rituximab. Again animals were inoculated with BJAB cells and after 16 days (when tumors became palpable and corresponding to week -1 relative to rituximab treatment), half of the animals were i.p. treated every 2 days with either 125 μg of CCL3(11–70) antagonist or control saline. One week later (week 0), when the tumor size had reached nearly 200 mm^2, groups of five antagonist-treated and control animals were given the standard dose of rituximab. Antagonist treatment was continued further for 1 wk after initiating rituximab and was stopped at week 1 (Fig. 4, gray bar). In contrast, rituximab treatment was continued with two other infusions on weeks 1 and 2 (Fig. 4, open arrows). Tumor growth in all animals was registered every 2 days throughout the experiment. The results confirmed that antagonist treatment did not affect tumor growth in the absence of rituximab treatment because tumor growth curves for all animal groups were superimposable up to week 0 (the beginning of rituximab treatment). In contrast, repeated inoculation with CCL3(11–70) completely abolished the therapeutic activity of rituximab. Indeed, although control animals treated with rituximab and mock saline showed rapid tumor disappearance, already complete 7–10 days following rituximab inoculation, the antagonist-treated animals did not respond to rituximab treatment but showed continued tumor growth during the same time period (week 0 to 1), overlapping with that of untreated mice (Fig. 4). Study of growth curves at later time points, i.e., after antagonist treatment had been interrupted, demonstrated that the inhibition by CCL3(11–70) of the therapeutic activity of rituximab is reversible. Indeed the second and third rituximab inoculations, performed after antagonist treatment were stopped, led to gradual reduction of the tumor mass over the following 2-wk period. Altogether these data demonstrate that CCL3(11–70) antagonist treatment dramatically inhibits the therapeutic activity of rituximab in vivo and that the effect is reversible.

The therapeutic activity of rituximab also requires immune cells for tumor eradication

The inhibitory effects of CCL3(11–70) antagonist on the therapeutic activity of rituximab suggested that β-chemokines, either upregulated directly by rituximab or induced during the inflammatory reaction triggered by the Ab, recruit and/or activate immune effector cells that then eradicate the tumor. To identify the immune effector cells involved in lymphoma control following rituximab inoculation, we analyzed the effect of depleting different leukocyte populations in vivo. NK cells or neutrophils were depleted by repeated treatment with the TM-β1 and RB6-8C5 Abs, respectively, starting 2 days before rituximab treatment, as previously described (11). As shown in Fig. 5, *A* and *B*, depletion of either NK cells or PMN cells did not affect tumor growth in absence of rituximab but essentially abolished the therapeutic activity of the Ab ($p < 0.0001$). To verify that the loss of rituximab activity was not an artifact due to the repeated treatments with anti-NK Abs, we also tested the role of NK cells by using Rag2$^{-/-}$c$\gamma^{-/-}$ mice, which completely lack NK cells as well as lymphocytes. These mice were s.c. inoculated with the standard doses of BJAB cells and were

then treated with rituximab as before. As shown in Fig. 5*C*, rituximab was completely inactive in the double knockout mice, confirming the requirement for NK cells by an independent method.

The CCL3 receptors CCR5 and CCR1 are also expressed by macrophages (40). We therefore also analyzed the role of macrophages by repeated treatments with clodronate-liposomes or PBS-liposomes as control, starting 2 days before rituximab treatment. As shown in Fig. 5*D*, depletion of macrophages also led to loss of the therapeutic activity of rituximab.

The data presented demonstrate that different immune effector cells are required to eradicate the bulky BJAB tumor following complement activation and CCL3/4 induction. We therefore also investigated whether CCL3(11–70) antagonist treatment modified immune cell infiltration into the tumor, either in the presence or absence of rituximab treatment. For this purpose, antagonist- or rituximab-treated or control animals were sacrificed at different times after the first rituximab treatment, and infiltration of NK cells, PMN cells, and macrophages within the tumor were analyzed by immunohistochemistry using the TM-β1, RB6-8C5, and F480 Abs, respectively. Cellular infiltration was quantified by semi-automated counting of stained cells. Infiltration of tumor with NK cells, macrophages, and PMN cells could be observed by immunohistochemistry in untreated tumors. In two separate experiments, rituximab treatment did not induce a reproducible increase in immune cell infiltrate in the tumor 4, 8, 24, 48, or 72 h after treatment. Macrophages were particularly abundant in the tumor and could not be observed to increase or decrease. PMN and NK cells were also clearly infiltrating the tumor even in the absence of Ab treatment, but again, their overall number was not reproducibly modified after rituximab inoculation (data not shown). Similarly we could not detect a reproducible and quantitative effect of antagonist treatment on tumor cell infiltration either in rituximab-treated or untreated animals at 8 or 24 h after the first rituximab treatment, for any of the populations analyzed (data not shown). These data suggest that the dramatic effects of both rituximab and CCL3(11–70) antagonist occur without macroscopic differences in immune cell recruitment within the tumor, at least within the time period examined.

Discussion

In this article, we demonstrate that rituximab induces CCL3 and CCL4 mRNA and protein expression in lymphoma cells in vitro and in vivo and suggest that the CCL3 family of chemokines are involved in the therapeutic activity of rituximab. Induction of CCL3 and CCL4 in BJAB cells in vitro was demonstrated both at the RNA and protein level, and chemokines produced were shown to be functional in chemotaxis assays. CCL3/4 induction was specific, and the $F(ab')_2$ of rituximab also up-regulated CCL3/4 suggesting that signaling through the CD20 molecule and not FcγR was responsible for chemokine induction. CCL3 RNA and protein were more highly expressed in BJAB cells than CCL4, and was demonstrated in vivo. We have previously shown by microarray analysis that a very restricted set of genes are up-regulated by CD20-mediated signaling in B lymphoma cells (16). Indeed other chemokines analyzed were not modulated by rituximab. Study of other B lymphoma lines showed that rituximab induced CCL3/4 in four of six lymphoma lines studied (data not shown) (16). For example CCL3 mRNA was induced 20-fold in EsIII cells but was not up-regulated in DHL-4 or Raji cells (16). Similarly, we have shown that CCL3/4 protein induction can be observed in some but not all freshly isolated lymphoma/leukemia samples (2/3). These results therefore demonstrate a heterogeneity of CCL3/4 induction also in naturally occurring human lymphomas. Whether such heterogeneity has an impact on the response of patients to rituximab in vivo is an intriguing hypothesis that deserves to be further investigated.

To further study the role of CCL3/4 in vivo, we set up and characterized a s.c. xenograft lymphoma model in athymic mice using BJAB cells. In this model, rituximab was able to eradicate the tumor within 2–3 wk, even though the BJAB lymphoma was relatively bulky because it was allowed to grow for 3–4 wk until it reached ~200–250 mm^2 before Ab treatment. Cured animals could be further kept for 3 mo without showing recurrence of tumor. CVF treatment of BJAB-inoculated animals suggested that complement is at least in part required for the therapeutic activity of rituximab also in this model, in agreement with our previous reports and reports from other groups using either CVF (12, 13) or $C1q^{-/-}$ animals (11). Also in human models, current evidence suggests that complement activation is an early event following rituximab administration to lymphoma patients, detected by the rapid release of C3, C4, and C5 complement fragments, which precedes cytokine release (41, 42). Other reports suggest that the cellular microenvironment may determine the extent of complement dependency of B cell clearance in mice (10).

To study the role of CCL3 in this model, we have synthesized and functionally characterized CCL3 antagonist CCL3(11–70), composed of the wild-type protein truncated at the N terminus. CCL3(11–70) showed no chemotactic activity in vitro but inhibited CCL3-induced chemotaxis. Several analogs of the β-chemokine family with modified or truncated N-termini have been described to have antagonist activity for the parent chemokine in vitro and in vivo (17, 43–45). These data therefore suggest that for CCL3, the N terminus also has an important role for receptor signaling. Most interestingly, the CCL3(11–70) antagonist showed a dramatic effect on the therapeutic activity of rituximab in vivo in the BJAB xenograft model. Although CCL3(11–70) had no effect on tumor growth in the absence of rituximab, it completely abolished the capacity of the Ab to eradicate the lymphoma. This effect was reversible because interruption of antagonist treatment was able to restore the therapeutic activity of the Ab. We reasoned that such a dramatic effect of antagonist treatment was likely due to interference with the recruitment and/or activation of immune cells required to kill tumor cells following rituximab administration. Indeed antagonist was able to inhibit the migration of monocytes in response to rituximab-stimulated BJAB supernatant in vitro. In vivo, depletion of different immune cell populations, including NK cells, polymorphonuclear cells, and macrophages, demonstrated that these cells are required for tumor eradication by rituximab. The role of NK cells was confirmed using $Rag2^{-/-}c\gamma^{-/-}$ animals that completely lack NK cells, and in which rituximab was indeed inactive. Thus CCL3 is likely to act through activation/recruitment of immune cells. A role for the macrophage phagocyte network has also been described by others using mouse anti-CD20 mAbs (46). In this latter case, however, complement did not seem to be required. These differences may be due to the different Abs used, and perhaps to the bulk of tumor cells in our study. Interestingly Hamaguchi et al. (46) observed improved peritoneal cavity B cell depletion after inflammation elicited effector cell migration into this body location. In the bulky BJAB tumor model described in this study, different immune cell populations cooperate with each other in eradicating the tumor. A similar combined activity of different mechanisms and different effector cells in the mechanism of action of rituximab has also been described by others (8, 10). Furthermore, the cooperation with immune cells, complement, and inflammatory mediators suggested in this study is reminiscent of that observed in Ab-mediated autoimmune diseases (47). A synergistic

effect of different immune cell types could also be explained by induction of inflammatory cytokine/chemokine cascades functionally linking the different immune cells (21, 28, 48).

In view of the evidence for a role of immune cells in the therapeutic activity of rituximab in this model, we also investigated whether rituximab or CCL3 antagonist modified the infiltration of these cells in the tumor. We could not detect any significant and reproducible effect of rituximab on infiltration by macrophages, NK cells, or neutrophils at 4–72 h after Ab administration. This result may have been due to the fact that these cells were already present in the lymphoma in significant numbers in the absence of rituximab treatment. Similarly, antagonist treatment did not modify reproducibly the number of infiltrating cells. We hypothesize therefore that rituximab and antagonist may affect the activation of immune cells, the cross-talks between different cell types, or their fine movements within the tumor rather their actual macroscopic recruitment within the tissue (20). It is worth noting that the inflammatory response to rituximab triggered by complement activation and its interaction with FcγR on immune cells is likely to induce production of CCL3 and other inflammatory CC chemokines by immune cells within the tumor, in particular infiltrating macrophages, as already demonstrated in different models of immune complex-mediated tissue injury (49–51). Furthermore, the two major receptors for CCL3 are CCR1 and CCR5, which also bind other molecules of the β-chemokine family, including CCL4, CCL5 (RANTES), as well as other molecules (44, 52, 53). Thus the CCL3(11–70) antagonist is likely to inhibit the activity of several chemokines in vivo, produced both by tumor and immune-infiltrating cells. This hypothesis together with a better definition of the role of CCR1 and CCR5 in the therapeutic activity of rituximab in vivo should be further investigated and is beyond the scope of this article.

Evidence for an important antitumor activity of CCL3 as well as other chemokines has been presented previously (reviewed in Ref. 54) in different in vivo tumor models (20–23, 55–58). However these reports mostly involve exogenous chemokines overexpressed through their direct inoculation or through gene transfer into the tumor cells. In the BJAB model presented in this study in contrast, we have shown a fundamental role for endogenously expressed chemokine. Furthermore, in contrast with some of these reports, CCL3 antagonist inhibited the therapeutic activity of rituximab but had no effect on spontaneous tumor growth in the absence of Ab treatment. Thus the antagonist effect was specific for the response to rituximab treatment. This observation is similar to some other published results showing antitumor effects of chemokines only in combination with other factors, such as drug-induced tumor destruction or coadministered cytokines (21, 54).

The data presented suggest that in this rather bulky BJAB tumor model, the combination of different immune-mediated mechanisms is necessary for tumor control. This suggestion may not be so surprising given the known interplay between complement activation, inflammation, and immune cells (15, 59). Indeed rituximab binding to its target triggers rapid complement activation that initiates a cascade of events that includes release of proinflammatory factors, C3a and C5a (41, 42). Secondly rituximab binding to CD20 induces CCL3 and CCL4 expression by lymphoma cells as shown in this study. These may contribute to immune effector cell activation. Finally, rituximab signaling through FcγR may also lead to release of proinflammatory cytokines such as TNF-α and IL-6, as described by other reports (41, 60), which may further amplify the immune response (61, 62) for optimal tumor removal by phagocytosis or Ab-dependent cytotoxicity. This network, in addition to inducing maximal immune function, may also create interdependency of different immune cell types, leading to the observed need for all cell types for the effective eradication of tumor.

These data altogether suggest that individual differences in expression of the β-chemokine family of proteins, either by the tumor itself or by infiltrating immune cells, may play a role in the clinical response of different patients to rituximab. Interestingly CCL3 was not expressed or induced in Raji cells, and rituximab was less active against the Raji than BJAB s.c. tumor (E. Cittera, unpublished observation). Also, differences in expression of CCL3 in human lymphomas have been described that in part predict patients' prognosis (63). Recently a genetic variation in the copy number of the homologous human genes CCL3-L1 and CCL4-L1 has been described that leads to variable protein expression levels (64). Further investigation of the role of CCL3 in the response of lymphoma patients to rituximab is therefore warranted.

In summary, this study shows rituximab induces the CCL3 and CCL4 β-chemokines in B lymphoma cells. Furthermore, it demonstrates that β-chemokines and complement are required for the therapeutic activity of rituximab in a bulky lymphoma xenograft model, and may cooperate in the recruitment/activation of the innate immunity network.

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Disclosures

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