The *in vivo* mechanism of action of CD20 monoclonal antibodies depends on local tumor burden

Peter Boross,[1] J.H. Marco Jansen,[1] Simone de Haij,[2] Frank J. Beurskens,[2] Cees E. van der Poel,[1] Lisette Bevaart,[1] Maaike Nederend,[1] Josée Golay,[3] Jan G.J. van de Winkel,[1,2] Paul W.H.I. Parren,[2] and Jeanette H.W. Leusen[1]

[1]Immunotherapy Laboratory, Department of Immunology, University Medical Center Utrecht, The Netherlands; [2]Genmab, Utrecht, The Netherlands, and [3]USC Hematology, Ospedali Riuniti di Bergamo, Bergamo, Italy

ABSTRACT

Background
CD20 monoclonal antibodies are widely used in clinical practice. Antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and direct cell death have been suggested to be important effector functions for CD20 antibodies. However, their specific contributions to the *in vivo* mechanism of action of CD20 immunotherapy have not been well defined.

Design and Methods
Here we studied the *in vivo* mechanism of action of type I (rituximab and ofatumumab) and type II (HuMab-11B8) CD20 antibodies in a peritoneal, syngeneic, mouse model with EL4-CD20 cells using low and high tumor burden.

Results
Interestingly, we observed striking differences in the *in vivo* mechanism of action of CD20 antibodies dependent on tumor load. In conditions of low tumor burden, complement was sufficient for tumor killing both for type I and type II CD20 antibodies. In contrast, in conditions of high tumor burden, activating FcγR (specifically FcγRIII), active complement and complement receptor 3 were all essential for tumor killing. Our data suggest that complement-enhanced antibody-dependent cellular cytotoxicity may critically affect tumor killing by CD20 antibodies *in vivo*. The type II CD20 antibody 11B8, which is a poor inducer of complement activation, was ineffective against high tumor burden.

Conclusions
Tumor burden affects the *in vivo* mechanism of action of CD20 antibodies. Low tumor load can be eliminated by complement alone, whereas elimination of high tumor load requires multiple effector mechanisms.

Key words: CD20 monoclonal antibodies, mechanism of action, local tumor burden.

Citation: Boross P, Jansen JHM, de Haij S, Beurskens FJ, van der Poel CE, Bevaart L, Nederend M, Golay J, van de Winkel JGJ, Parren PWHI, and Leusen JHW. The in vivo *mechanism of action of CD20 monoclonal antibodies depends on local tumor burden. Haematologica 2011; 96(12):1822-1830. doi:10.3324/haematol.2011.047159*

PB and JHMJ contributed equally to this manuscript.

Acknowledgments: the authors would like to thank Marleen Voorhorst for technical assistance and Piet Aerts for providing mouse anti-rabbit serum for the hemolytic complement assay and Drs Linde Meyaard and Erik Hack for critically reading the manuscript.

Funding: this research was supported by the following grants: Association for International Cancer Research grant 06-0368, Dutch Cancer Foundation (KWF/NKB) grant UU2002-2706, and Dutch Science Fund (NWOALW2PJ/05 088). JHMJ was financially supported by Genmab B.V.

Manuscript received on May 9, 2011. Revised version arrived on July 27, 2011. Manuscript accepted on August 24, 2011.

Correspondence: Jeanette H.W. Leusen, Immunotherapy Laboratory KC 02.085.2, Department of Immunology, Lundlaan 6, 3584 EA Utrecht, The Netherlands. Phone: international +31.88.7554268. Fax: international +31.88.7554305. E-mail: jleusen@umcutrecht.nl

The online version of this article has a Supplementary Appendix.

Introduction

CD20 monoclonal antibodies are widely used to treat follicular or low-grade non-Hodgkin's lymphoma and chronic lymphocytic leukemia as well as various autoimmune conditions, such as rheumatoid arthritis.[1,2] Based on *in vitro* characterization, two types of CD20 monoclonal antibodies are recognized: type I are able to relocate CD20 molecules into lipid microdomains and efficiently activate complement, whereas type II promote strong homotypic adhesion and induce apoptosis. Both types of CD20 monoclonal antibodies can mediate antibody-dependent cellular cytotoxicity (ADCC).[3] The success of rituximab has stimulated the development of second-generation (human) and third-generation (Fc-engineered) CD20 monoclonal antibodies to further improve therapeutic performance. Ofatumumab is a novel human type I IgG1 CD20 monoclonal antibody, which binds to a distinct membrane proximal epitope encompassing both the small and large loops on the CD20 molecule.[4-6] Ofatumumab activates complement more efficiently than rituximab, most likely because of its distinct binding characteristics.[5] Ofatumumab was recently approved for the treatment of fludarabine- and alemtuzumab-refractory chronic lymphocytic leukemia in the USA by the Food and Drug Administration and in Europe by the European Medicines Agency.[7,8] A typical example of type II CD20 monoclonal antibodies is B1, a murine monoclonal antibody that is currently used in the clinic in a radio-labeled form (tositumomab), and that may also be very effective in a non-conjugated form, at least in *in vivo* mouse tumor models.[9]

CD20 monoclonal antibodies can engage multiple effector mechanisms *in vivo* for the elimination of tumor cells; however, their relative importance and contribution to the *in vivo* mechanism of action is still not fully understood.[3] Target binding of CD20 monoclonal antibodies can result in direct cytotoxicity.[10,11] *In vitro* studies suggested that this effect can be enhanced by secondary cross-linking,[12] but we have recently shown that this mechanisms is unlikely to contribute to the *in vivo* mode of action of CD20 monoclonal antibodies.[13] A large body of evidence based on both clinical and preclinical studies supports a role for ADCC through interaction of rituximab-opsonized CD20-positive cells with FcγR-expressing effector cells,[14-20] in particular, monocytes and macrophages.[17,21] The role of complement in rituximab immunotherapy is still under discussion. Whereas several *in vitro* and *in vivo* studies as well as observations in the clinic support its contribution,[22-24] other studies have found no role for complement[17] or have even shown detrimental effects in an *in vitro* ADCC assay.[25]

The efficacy of rituximab is influenced by a number of factors in patients, such as genetic variability in FcR,[15] level of CD20 expression,[26] intensity of CD20 internalization,[27] and human anti-chimeric antibody (HACA) titers. Tumor burden might also influence the efficacy of rituximab therapy by reducing available monoclonal antibodies in the circulation. In line with this, it was found in both mice and patients that rituximab serum concentrations are inversely correlated with tumor burden.[28-30] However, it is not known whether tumor burden also affects the *in vivo* mechanism of action of CD20 monoclonal antibodies. A better understanding of their *in vivo* mechanism of action will aid further optimization of the use of CD20 monoclonal antibodies in immunotherapeutic regimens and improve therapeutic success.

To investigate the impact of tumor burden on CD20 monoclonal antibody effector mechanisms we used a syngeneic, short term, *in vivo* tumor model. We compared two type I and one type II CD20 monoclonal antibodies using mouse EL4 tumor cells stably transduced with human CD20 (EL4-CD20). We studied tumor killing in the model under low and high tumor burden conditions and assessed the contribution of different effector functions of the CD20 monoclonal antibodies to their *in vivo* mechanism of action.

Design and Methods

Mice

C57Bl/6 mice were purchased from Janvier (Le Genest Saint Isle, France) or were bred in our facilities. Mac-1/CD11b-deficient mice (CR3$^{-/-}$) on the C57Bl/6 background were kindly provided by Dr T.N. Mayadas (Harvard Medical School, Boston, MA, USA). FcγRI$^{-/-}$, FcγRIIB$^{-/-}$, FcγRIII$^{-/-}$, and FcRγ$^{-/-}$ mice were bred and maintained at the SPF facility in the Central Animal Laboratory of Utrecht University. NOTAM mice express normal surface levels of FcγR but these are incapable of signaling due to mutations in the ITAM motif in the signal transducing FcRγ-chain.[13] All experiments were approved by the local animal ethical committee.

Cell culture

Mouse EL4 lymphoma cells stably transduced with human CD20 (EL4-CD20) were described previously.[22] Bone marrow-derived macrophages were cultured in the presence of 5 ng/mL GM-CSF (Cell Sciences) and culture medium was refreshed on days 2 and 5 as earlier described.[31] Adherent cells were used as bone marrow-derived macrophages on day 7–8. Macrophages from C57Bl/6 and CR3$^{-/-}$ mice for *in vitro* serum-enhanced ADCC experiments were cultured for 7-8 days in the presence of 40 ng/mL M-CSF (Peprotech).

Antibodies and flow cytometry

Ofatumumab (huIgG1, GlaxoSmithKline/Genmab), rituximab (chimeric IgG1, Roche) and 11B8 (HuMab-11B8, huIgG1, Genmab) were used as CD20 monoclonal antibodies. Bound CD20 monoclonal antibody was detected by FITC-labeled F(ab')2 fragments of goat anti-human IgG antibody (Protos Immunoresearch). Expression of FcγRI was determined using anti-mouse CD64 (clone X54-5/7.1.1, mIgG1, Pharmingen). Expression of FcγRIII was determined by blocking the cells with a FcγRIIB-specific monoclonal antibody followed by staining with anti-FcγRII/III (clone 2.4G2, ratIgG2b, Pharmingen). Antibodies against FcγRIV (clone 9E9, hamster IgG1) and FcγRIIB (clone K9.361, Ly17.2-specific) were kind gifts from Drs. Falk Nimmerjahn and Sjef Verbeek, respectively. FcγR expression was measured on F4/80^{+} (clone A3-1, ratIgG2b, Serotec)/ CD11b^{+} cells (clone M1/70, ratIgG2b, Pharmingen) in peritoneal lavage after blocking with 5% mouse serum.

Induction of annexin-V positivity and homotypic aggregation

EL4-CD20 cells were incubated for 30 min at 37°C with increasing concentrations of ofatumumab. Cross-linking was induced by 5 μg/mL F(ab')$_2$ fragments of rabbit anti-human IgG

antibody for 24 h. After 24 h the cells were stained with annexin-V RPE (Pharmingen) according to the manufacturer's protocol. For the induction of homotypic aggregation EL4-CD20 cells were incubated with 1 μg/mL ofatumumab or 11B8 for 24 h. Images were obtained using an EVOS light microscope.

Complement deposition

EL4-CD20 cells were incubated for 30 min at room temperature with increasing concentrations of CD20 monoclonal antobody. Unbound CD20 monoclonal antobody was washed away and pooled C57Bl/6 serum (n=3) was added at a concentration of 0.5%, and incubated for 45 min at 37°C. Complement deposition was measured using anti-mouse C3b/iC3b/C3c (clone 3/26, rat IgG2a, Hycult Biotech) followed by secondary polyclonal goat anti-rat Ig RPE (Pharmingen). Although we took all the precautions to conserve full complement activity as suggested by Lachmann,[32] we were not able to measure CDC by either type I or II CD20 monoclonal antobody *in vitro* using the EL4-CD20 cells and fresh mouse serum.

Macrophage killing assay

Bone marrow-derived macrophages were plated together with 10,000 CFSE-labeled CD20 monoclonal antobody-opsonized EL4-CD20 cells at different effector-to-target (E:T) ratios. After overnight incubation, cells were trypsinized and the numbers of CFSE positive cells were determined relative to the constant amount of beads (Molecular Probes, Invitrogen). For complement-enhanced killing, CD20 monoclonal antobody-opsonized CFSE-labeled EL4-CD20 cells were incubated with 5% mouse serum, heat-inactivated (56°C for 30 min) mouse serum or serum from cobra venom factor (CVF)-treated mice for 45 min at 37°C prior to adding bone marrow-derived macrophages. The number of tumor cells in wells without antibody was set at 100%. Relative killing was calculated as the decrease in the number of tumor cells in the presence of CD20 monoclonal antobody relative to conditions without antibody.

EL4-CD20 lymphoma model

Mice were injected intraperitoneally with 5×10^5 or 5×10^6 CFSE-labeled EL4-CD20 lymphoma cells as low and high tumor burden, respectively, and 16 h later were given rituximab, ofatumumab, 11B8 or 100 μL phosphate-buffered saline intraperitoneally. The peritoneal cavity was washed with phosphate-buffered saline containing 5 mM EDTA 24 h after antibody injection and the total number of tumor cells was determined using TruCount tubes (BD Biosciences). Tumor cells remained in the peritoneal cavity, as we did not find tumor cells in the circulation or in the spleen and bone marrow at the time of lavage (*data not shown*).

Complement depletion

Complement (C3/C5) was depleted by injecting 2 times 10 U CVF (*Naja naja kaouthia*, Quidel) intraperitoneally 1 day prior to injection of tumor cells. Serum from CVF-treated mice was not able to induce deposition of complement fragments C3b, iC3b and C3c *in vitro* when incubated with ofatumumab-opsonized EL4-CD20 cells. In addition, serum from CVF-treated mice was found to be negative in an overall mouse serum hemolytic complement activity assay[33] indicating that complement was fully inactivated (*data not shown*).

Statistics

Parametric data are represented as mean ± SEM and have been analyzed for significance using Student's unpaired t-test. Multiple groups in the *in vivo* experiments were compared by analysis of variance and further evaluated using Bonferroni's multiple comparison test using Graph Pad 4.0 software. A P value of 0.05 or less is considered statistically significant.

Results

Effector mechanisms of CD20 monoclonal antibody in vitro with EL4-CD20 target cells

For our *in vivo* studies, we wished to employ syngeneic mouse EL4 lymphoma cells that homogeneously express human CD20.[22] Because, it is not known whether EL4-CD20 cells are sensitive to killing by the different CD20 monoclonal antibody effector mechanisms, we first validated EL4-CD20 cells *in vitro* using the type I CD20 monoclonal antibodies rituximab and ofatumumab and the type II CD20 monoclonal antibody 11B8.[4]

In accordance with previous studies employing B-cell lines and primary lymphomas, type I CD20 monoclonal antibodies exhibited approximately two-fold higher binding to EL4-CD20 cells than type II CD20 monoclonal antibodies (*Online Supplementary Figure S1A*).[2,4] Cross-linking of bound CD20 monoclonal antibody induces direct cell death in lymphoma cell lines.[12] In line with this, cross-linking of CD20 monoclonal antibody on EL4-CD20 cells with anti-human IgG antibody induced annexin-V staining (*Online Supplementary Figure S1B and data not shown*). In the absence of cross-linking antibody, only 11B8 was able to induce homotypic aggregation of EL4-CD20 cells (*Online Supplementary Figure S1C* and *data not shown)*.

Ofatumumab and rituximab binding induced deposition of complement fragments on EL4-CD20 cells, whereas 11B8 did so, and only modestly at high CD20 monoclonal antibody concentrations (*Online Supplementary Figure S1D*). Ofatumumab and rituximab were more efficient than 11B8 in an *in vitro* killing assay using bone marrow-derived macrophages (*Online Supplementary Figure S1E*), confirming published data derived from using human effector cells and primary lymphoma cell lines.[4] Ofatumumab mediated significantly better tumor killing than rituximab at lower CD20 monoclonal antibody concentrations (0.01 μg/mL) (*Online Supplementary Figure S1E*) (**P<0.01; ANOVA).

Taken together, these *in vitro* results show that EL4-CD20 cells can be used as target cells to study CD20 monoclonal antibody mechanisms *in vivo*. Indeed, the effector mechanisms that CD20 monoclonal antibodies employ to kill EL4-CD20 cells are in agreement with earlier data using B cell lines and primary lymphomas.

Differential involvement of FcγR under low and high tumor burden conditions

To study the effect of tumor burden on the CD20 monoclonal antibody effector mechanisms *in vivo*, we used a peritoneal, syngeneic, tumor model as described earlier.[13] EL4-CD20 cells were injected intraperitoneally followed by injection of CD20 monoclonal antibody at the same location 16 h later. The number of tumor cells was determined 24 h after injection of antibody. We injected 5×10^5 and 5×10^6 EL4-CD20 cells to establish low and high tumor burden conditions, respectively. Five-hundred thousand EL4-CD20 cells was the minimal number of cells we could inject for reliable analysis at the end of the experiment at which, on average, we recovered 3.9×10^5 cells in mice treated with phosphate-buffered saline or control anti-

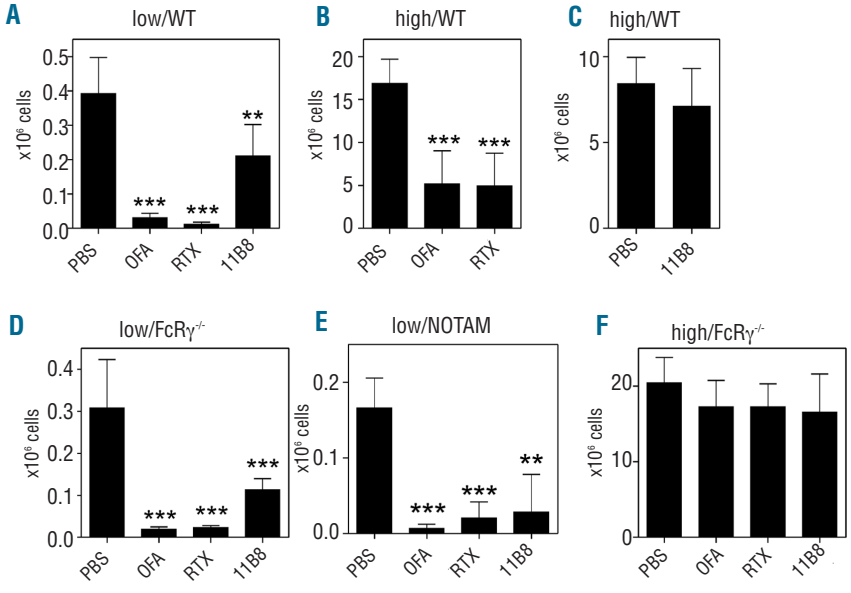

Figure 1. Differential requirement for FcγR for tumor killing by CD20 monoclonal antibodies in the low and high tumor burden models. EL4-CD20 intraperitoneal (i.p.) model: mice were injected i.p. with CFSE-labeled EL4-CD20 cells and 16 h later were given CD20 monoclonal antibodies (mAb) or phosphate buffered saline (PBS). After 24 h the number of tumor cells in the peritoneal wash was determined by TrueCount tubes (4-5 mice/group; ***$P<0.001$, **$P<0.01$; ANOVA). (A) Low tumor burden model: wild-type (WT) mice were injected with 5x10⁵ EL4-CD20 cells and 10 μg ofatumumab (OFA), rituximab (RTX), 11B8 or PBS. (B) and (C) High tumor burden model: WT mice were injected with 5x10⁶ EL4-CD20 cells and 100 μg CD20 mAb or PBS alone. FcRγ-/- (D) and NOTAM (E) mice were injected with 5x10⁵ EL4-CD20 cells and 10 μg CD20 mAb or PBS alone. (F) FcRγ-/- mice were injected with 5x10⁶ EL4-CD20 cells and 100 μg CD20 mAb or PBS alone.

body; this we considered the low tumor burden condition. Treatment with 10 μg ofatumumab or rituximab effectively reduced the number of tumor cells, whereas the effect of 11B8 was less prominent (Figure 1A and *Online Supplementary Table S1*). Tumor killing was also observed with 1 μg ofatumumab but not with 0.1 μg (*Online Supplementary Figure S2A*). We did not observe killing of EL4-CD20 cells by nonspecific human IgG1 (*Online Supplementary Figure S2A*) or killing of CD20 negative EL4 cells (*data not shown*).

Five million EL4-CD20 cells was the largest number of cells we could inject and still observe a treatment effect. At the end of the experiment, from the mice treated with phosphate-buffered saline, we recovered on average 17.76×10⁶ EL4-CD20 cells indicating that at least part of the cells had divided *in vivo*; this we considered the high tumor burden condition. Injection of 100 μg ofatumumab or rituximab significantly reduced the amount of recovered tumor cells (Figure 1 B-C and *Online Supplementary Table S1*). Significant tumor killing was observed in wild-type mice by ofatumumab and rituximab using 10 μg and 3 μg monoclonal antibody but not at 1 μg (*Online Supplementary Figure S2B,C*). Type II CD20 monoclonal antibody 11B8 was not effective in tumor killing at any of the concentrations tested (Figure 1C and *Online Supplementary Figure S2D*). Injection of 10 and 100 μg CD20 monoclonal antibody in the model under low and the high tumor burden conditions, respectively, resulted in saturating concentrations as detected by the inability to stain free CD20 on recovered tumor cells (*data not shown*).

ADCC via FcγR is an important effector mechanism of CD20 monoclonal antibodies;[19] we, therefore, first assessed the role of FcγR. To this end we used FcRγ-/- mice that lack all activating FcγR and NOTAM mice that express normal surface levels of signaling-deficient FcγR in our model with low and high tumor burden.[13] The efficacy of CD20 monoclonal antibodies against low tumor burden was not affected in FcRγ-/- mice (Figure 1D) or in NOTAM mice (Figure 1E). In contrast, under high tumor burden,

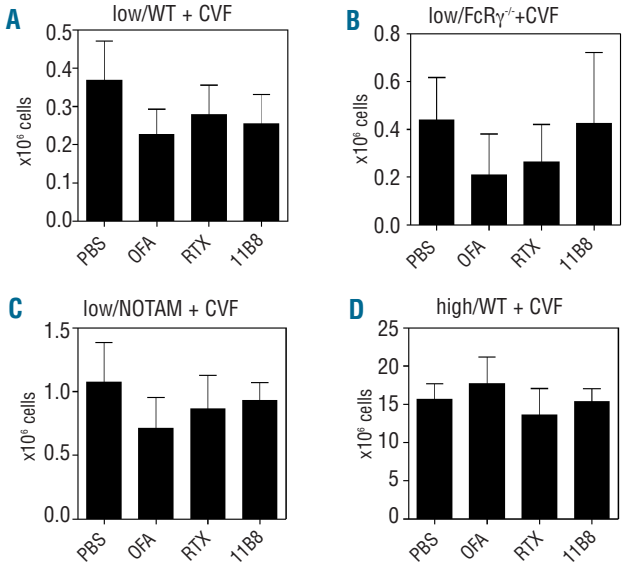

Figure 2. Complement is essential for tumor killing by CD20 monoclonal antibody (mAb) at both low and high tumor burden. EL4-CD20 intraperitoneal (i.p.) model: mice were injected i.p. with CFSE-labeled EL4-CD20 cells and 16 h later were given CD20 mAb or phosphate buffered saline (PBS). After 24 h the number of tumor cells in a peritoneal wash was determined using TrueCount tubes. Complement was depleted with cobra venom factor (CVF) 1 day prior to the injection of tumor cells (3-5 mice/group). CVF-treated wild-type (WT) (A), FcRγ-/- (B) or NOTAM mice (C) were injected with 5x10⁵ EL4-CD20 cells and 10 μg OFA, RTX, 11B8 or PBS alone. (D) CVF-treated WT mice were injected with 5x10⁶ CFSE-labeled EL4-CD20 cells and 100 μg ofatumumab (OFA), rituximab (RTX), 11B8 or PBS.

CD20 monoclonal antibody therapy by rituximab or ofatumumab was fully abrogated in FcRγ-/- mice (Figure 1F) and in NOTAM mice.[13] This indicates that FcγR are essentially required for tumor killing under the high but not under the low tumor burden conditions in our model.

Complement is required for killing of tumor cells by CD20 monoclonal antibodies at both low and high tumor burden

Complement activation may constitute an important effector mechanism of CD20 monoclonal antibodies.[22,24,34] We, therefore, depleted complement using CVF prior to the injection of tumor cells. CVF treatment abrogated CD20 monoclonal antibody therapy in WT mice under low tumor burden conditions in wild type, FcRγ$^{-/-}$ and NOTAM mice (Figures 2 A-C). Interestingly, CD20 monoclonal antibodies also required complement under the high tumor burden condition as depletion of complement also fully abrogated therapy (Figure 2D).

Taken together these results show that complement is sufficient for the elimination of tumor cells by both type I and type II CD20 monoclonal antibodies at low tumor burden. In contrast, both FcγR and complement seem to be necessary for tumor killing in the high tumor burden model.

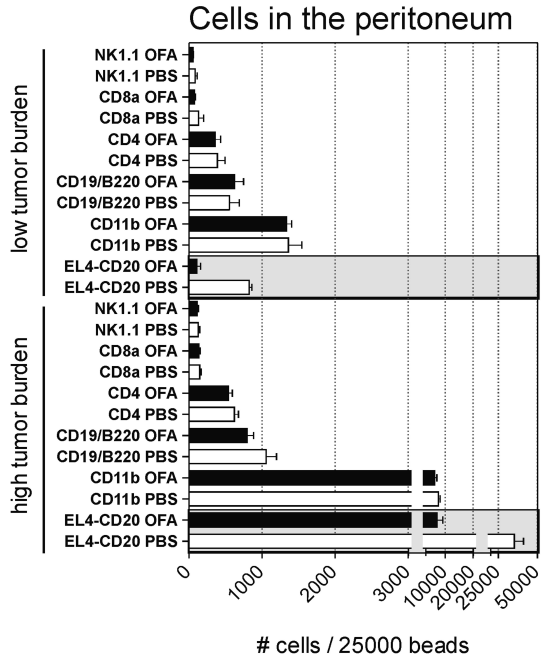

Figure 3. Distribution of cell populations in the peritoneal cavity following tumor challenge and treatment. The relative numbers of tumor cells and different effector cell populations in the peritoneum at the end of experiments carried out with low and high tumor burden as determined by flow cytometry using fixed amount of beads. The effector cell populations and markers used for identification were as follows: NK cells (NK1.1), CD8$^+$ T cells (CD8α), CD4$^+$ T cells (CD4), B cells (CD19/B220), macrophages (CD11b).

Dominant role for FcγRIII on macrophages at high tumor burden

Monocytes and macrophages, rather than neutrophils and NK cells, have been shown to be important effector cells in CD20 monoclonal antibody therapy in mice.[17,21] In line with this, the majority of effector cells in the peritoneal cavity were found to be F4/80$^+$CD11b$^+$ FcγR-expressing mononuclear cells (Figure 3). We found negligible numbers of NK cells (defined as NK1.1$^+$) and no polymorphonuclear cells in the peritoneal cavity even after 6 or 12 h following CD20 monoclonal antibody injection (Figure 3 and *data not shown*).

We observed efficient tumor killing *in vitro* by macrophages (*Online Supplementary Figure S1E*), but bone marrow polymorphonuclear cells or pegylated granulocyte colony stimulating factor-stimulated peripheral blood polymorphonuclear cells were unable to kill opsonized EL4-CD20 cells (*data not shown*). These data strongly suggest that in our model with high tumor burden the cells involved in the elimination of EL4-CD20 cells are macrophages, which is in accordance with earlier findings.[17,21,35,36]

Consistent with literature data, peritoneal F4/80$^+$ macrophages expressed all four FcγR in mice: the activat-

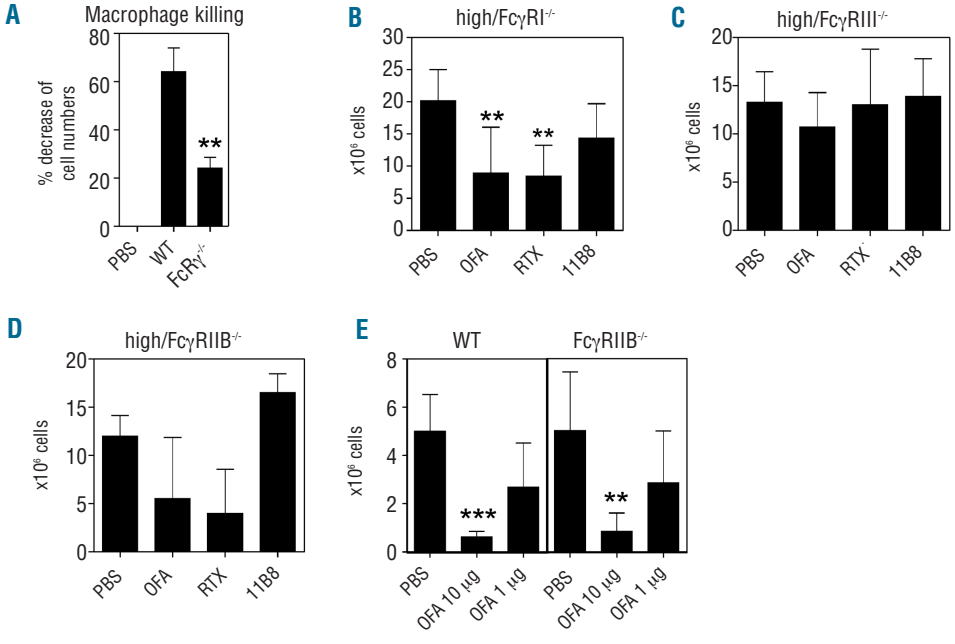

Figure 4. Dominant role for FcγRIII in tumor killing at high tumor burden. (A) *In vitro* macrophage killing with wild-type (WT) and FcRγ$^{-/-}$ macrophages using 1 μg/mL ofatumumab (OFA). Data are representative of two independent experiments (**P<0.01; Student's t-test). EL4-CD20 intraperitoneal model: mice were injected i.p. with CFSE-labeled EL4-CD20 cells and 16 h later were given CD20 monoclonal antibody (mAb) or phosphate buffered saline (PBS). After 24 h the number of tumor cells in the peritoneal wash was determined using TrueCount tubes (3-10 mice/group; ***P<0.001, **P<0.01; ANOVA). FcγRI$^{-/-}$ (B), FcγRIII$^{-/-}$ (C), or FcγRIIB$^{-/-}$ (D) mice were injected with 5x10^6 EL4-CD20 cells and with 100 μg OFA, rituximab (RTX), 11B8 or PBS. (E) WT and FcγRIIB$^{-/-}$ mice were injected with 5x10^6 EL4-CD20 cells and with a suboptimal amount (10 and 1 μg) of OFA.

ing FcγRI, FcγRIII and FcγRIV and the inhibitory FcγRIIB (*Online Supplementary Figure S3A*).[37] However, FcγRIII expression is higher and FcγRIIB expression is somewhat lower on macrophages under high tumor burden conditions compared to low tumor burden conditions (*Online Supplementary Figure S3A*). This may reflect complement activation caused by the tumor cells without antibody. It is known that complement fragment C5a can tip the balance of activating and inhibiting FcγR on macrophages towards the activating FcγR.[38] In agreement with the *in vivo* results, *in vitro* tumor killing by FcRγ$^{-/-}$ macrophages, lacking expression of all activating FcγR, was significantly impaired compared to killing by WT macrophages (Figure 4A). The increased tumor killing by FcRγ$^{-/-}$ macrophages compared to PBS conditions is most likely mediated by non-specific receptors in an antibody-independent manner. Next, we assessed which individual FcγR is involved in the tumor killing *in vivo*. Interestingly, in contrast to our previous findings in a lung melanoma model, we observed normal tumor therapy in FcγRI$^{-/-}$ mice (Figure 4B).[39] In contrast, tumor killing was fully abrogated in FcγRIII$^{-/-}$ mice (Figure 4C), indicating a dominant role for FcγRIII in this model. Clynes *et al.* have shown that monoclonal antibody therapy is more efficient in mice deficient for the inhibitory FcγRIIB.[14] However, we did not observe increased tumor killing in FcγRIIB$^{-/-}$ mice (Figure 4D), even when suboptimal CD20 monoclonal antibody concentrations (10 and 1 μg) were used (Figure 4E).

Taken together, we identified a dominant role for mouse FcγRIII on macrophages in tumor killing in high tumor burden conditions.

Crucial role for complement-enhanced antibody-dependent cellular cytotoxicity via CR3 at high tumor burden

We have identified a co-dominant role for FcγR and complement in our model in high tumor burden conditions (Figures 1F and 2D). Of importance, we observed deposition of complement fragments (C3b, iC3b, C3c) on EL4-CD20 cells recovered from the peritoneal cavity from animals with high tumor burden, not only at 24 h (Figure 5A) but also after 6 and 12 h (*data not shown*) after CD20 monoclonal antibody injection. Deposited complement fragments can serve as ligands for complement receptors,

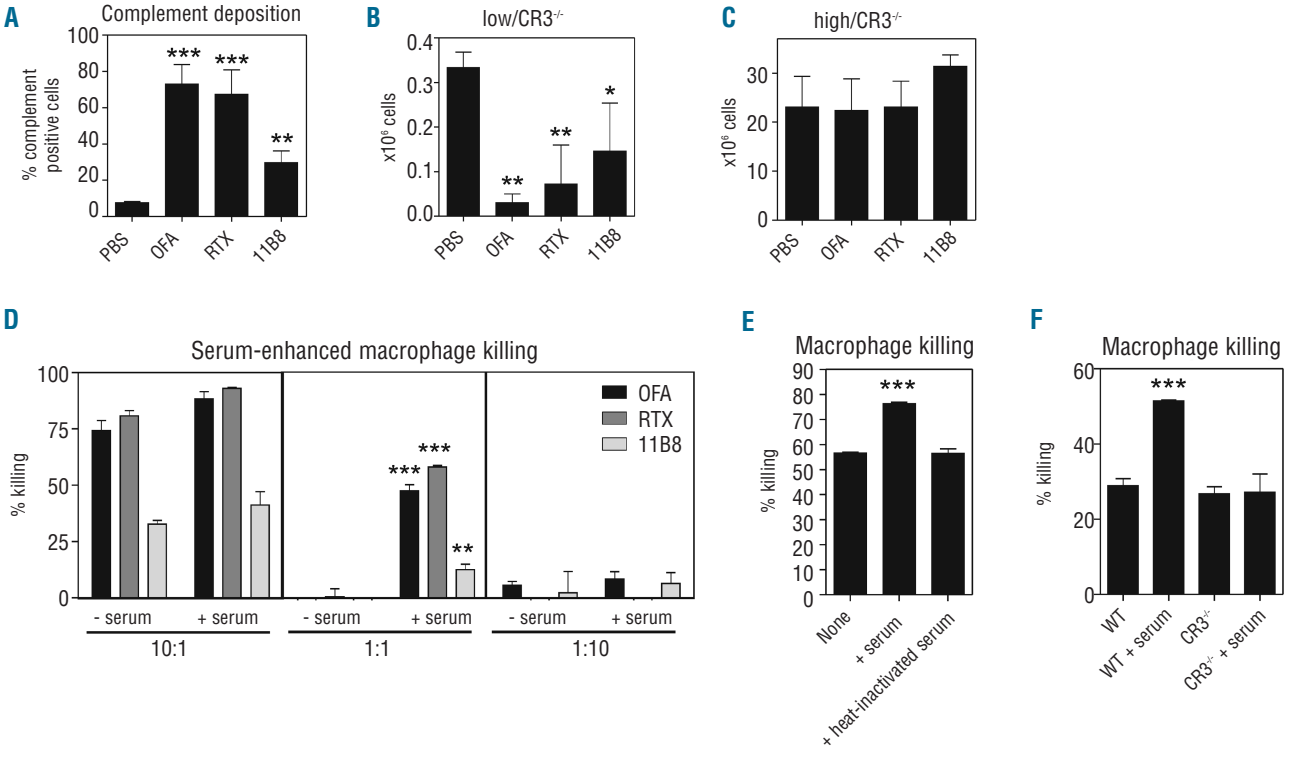

Figure 5. Deposited complement fragments enhance macrophage-mediated tumor killing via CR3. (A) Deposition of complement fragments C3b, iC3b and C3c on the tumor cells *in vivo*. EL4-CD20 intraperitoneal model: mice were injected i.p. with CFSE-labeled EL4-CD20 cells and 16 h later were given CD20 monoclonal antibody (mAb) or phosphate-buffered saline (PBS). After 24 h the number of tumor cells in the peritoneal wash was determined using TrueCount tubes (3-7 mice / group, ***P<0.001, **P<0.01, *P<0.05; ANOVA). (B) CR3$^{-/-}$ mice were injected with 5x10^5 EL4-CD20 cells (low tumor burden) and 10 μg CD20 mAb. (C) CR3$^{-/-}$ mice were injected with 5x10^6 CFSE-labeled EL4-CD20 cells (high tumor burden) and 100 μg ofatumumab (OFA), rituximab (RTX), 11B8 or PBS alone. (D) *In vitro* macrophage killing. CFSE-labeled EL4-CD20 cells opsonized in 1 μg/mL OFA, RTX and 11B8 were incubated for 24 h with bone marrow derived macrophages (BMDM) at different E:T ratios. Prior to adding BMDM, the CD20 mAb-opsonized CFSE-labeled EL4-CD20 cells were incubated with 5% wild-type (WT) mouse serum at 37°C. The number of CFSE-labeled EL4-CD20 cells was determined by using known amount of beads. The condition without CD20 mAb was used to set the 0% level; the condition without tumor cells was used to set the 100% level and killing is expressed as percentage decrease in cell numbers compared to control. (E) As in panel (D), *in vitro* macrophage killing with WT macrophages at E:T = 5:1 and 1 μg/mL RTX using heat-inactivated serum. (F) As in panel (D), *in vitro* macrophage killing with WT and CR3$^{-/-}$ macrophages at E:T = 3:1 and 1 μg/mL RTX). Data are representative of at least two experiments (***P<0.001, **P<0.01; Student's t-test).

CR3 and CR4, leading to complement-enhanced ADCC. To investigate the role of such interactions in our model we tested CR3$^{-/-}$ mice. Tumor killing at low tumor burden in CR3$^{-/-}$ mice was similar to that in WT mice (Figure 5B). Strikingly, tumor killing was completely abrogated in CR3$^{-/-}$ mice with high tumor burden (Figure 5C). The number of F4/80^{+} macrophages in the peritoneal cavity in CR3$^{-/-}$ mice was comparable to that in WT mice (*Online Supplementary Figure S3B*). Furthermore, addition of complement active serum in an *in vitro* macrophage killing assay significantly enhanced killing efficacy by both type I and type II CD20 monoclonal antibodies at suboptimal, (1:1) effector-to-target (E:T) ratios but not at E:T ratios of 10:1 or 1:10 (Figure 5D). The serum-enhancing effect was not observed when heat-inactivated serum or when CR3-deficient macrophages were used (Figure 5 E and F).

Summarizing, tumor killing by CD20 monoclonal antibodies both *in vivo* and *in vitro* can, therefore, be enhanced by recognition of deposited complement fragments on tumor cells by complement receptors expressed on macrophage effector cells.

Discussion

Here we investigated the effect of tumor burden on the *in vivo* mechanism of action of CD20 monoclonal antibodies in a short-term intraperitoneal syngeneic model. In contrast to systemic, disseminated lymphoma models, the peritoneal model offers the possibility of determining the local number of effector and tumor cells during monoclonal antibodies therapy and to study the effect of different CD20 monoclonal antibodies concentrations in a controlled manner. We opted for injection of 5×10^5 and 5×10^6 cells as low and high tumor burden, respectively. CD20 monoclonal antibody therapy reduced low tumor burden by approximately 95%, whereas an approximately 70% reduction was seen at high tumor burden. Although in absolute numbers a lower number of effector cells was recruited in the low tumor burden condition, compared to the high tumor burden, the *in vivo* effector-to-target cell ratios were estimated to be 7:1 and 1:1 for low and high tumor burdens, respectively (Figure 3). Importantly, *in vitro* macrophage killing was efficient at 7:1 but not at 1:1 effector-to-target ratios.

Complement and ADCC have been suggested to play a role in the *in vivo* mechanism of action of CD20 monoclonal antibodies, however, their relative importance is not well understood.[3] We found a prominent role for complement, which is in line with earlier observations using EL4-CD20 cells in an intravenous model.[22,23] In our model we used the peritoneal cavity as the tumor location. With respect to availability of complement proteins it has been reported that all complement components are present in the peritoneum[40] and that active complement deposition can be induced using normal peritoneal fluid.[41] Tumor reductions observed in the EL4-CD20 model with low tumor burden model could be explained by efficient CDC alone.

FcγR have been implicated in CD20 monoclonal antibody therapy in both mouse models and in patients.[16-18,42] Our results with the high tumor burden conditions confirm a role for FcγR, specifically FcγRIII. Unexpectedly, we did not find an inhibitory role for FcγRIIB. This may be explained by the skewed FcγR expression pattern under high tumor burden conditions. Although human IgG1 interacts well with both mouse FcγRIII and FcγRII (Overdijk *et al.* manuscript in preparation), the use of human antibodies is likely to change the relative importance of the FcγR used by mouse macrophages.

Interestingly, we found a role for CR3 at high tumor burden suggesting that under conditions of suboptimal effector-to-target ratios, complement-enhanced ADCC may substantially contribute to the anti-tumor mechanism of CD20 monoclonal antibodies. The importance of CR3 has been previously shown with solid tumor models.[43] Most likely, CR3 a enhances ADCC via stabilizing cytotoxic synapses as shown previously.[43,44] Synergy between FcR and complement in tumor killing may also occur at limiting antibody concentration or low antigen density as shown by antibody therapy of EL4 cells.[45]

It has been reported that CD20 monoclonal antobody therapy may temporarily exhaust the complement system[24,46-48] Certain anatomical locations, such as non-malignant peritoneal fluid (ascites) contain insufficient complement for the lysis of target cells. However, at these locations, activation of C3 and deposition of C3 fragments still occur resulting in "complement-marked" tumor cells.[49] Indeed, deposited complement fragments can be detected on circulating tumor cells during rituximab therapy in animal models and patients.[50] Tumor cells often upregulate complement regulatory proteins, mainly CD59, to evade direct complement-mediated lysis. CD59 blocks MAC assembly but tumor cells will be opsonized by complement fragments and therefore could be eliminated by CR3-dependent complement-enhanced ADCC.

We have identified macrophages as the most important effector cells, which is in line with earlier results.[17,21] Using an endogenous B-cell depletion model, it has been shown that different effector mechanisms are important in the depletion of distinct B-cell compartments. Circulatory B cells were found to be depleted by FcγR-expressing resident macrophages in the spleen and liver, whereas B cells or tumor cells with reduced circulatory capacity exhibited a greater dependence on complement-dependent mechanisms.[21] These results can partially be explained by different E:T ratios in the different compartments. Our model with non-circulating tumor cells confirms the crucial role of complement in the depletion of both tumor cells at low and high tumor burden.

Type I CD20 monoclonal antibodies showed superior anti-tumor effects compared to type II CD20 monoclonal antibodies in our model both at low and high tumor burden, which is most likely due to their better complement-activating properties. Ofatumumab is reported to activate complement more efficiently than rituximab.[6] We found ofatumumab to be superior to rituximab in *in vitro* ADCC by mouse macrophages. Interestingly, killing of cells under low tumor burden conditions with 11B8 was also dependent on complement activation. This is in line with recent findings that complement activation also affects type II CD20 monoclonal antibody tositumumab.[34] It should be noted, however, that EL4 cells, unlike most human tumor cells, are negative for the complement regulatory protein CD59 and, therefore, may show increased sensitivity to complement-mediated killing.[45] This may result in overestimation of the role of complement; nevertheless complement alone was clearly insufficient for killing under the high tumor burden conditions. Type II CD20 monoclonal antibodies are capable of inducing

direct cell death[11] and may give better anti-tumor effects, in certain models, than type I CD20 monoclonal antibodies.[9] The short time-scale of our model did not allow full assessment of therapeutic activity of 11B8.

In patients, it is currently not possible to accurately measure the mass of disseminated lymphomas and local effector-to-target ratios during rituximab therapy. Nevertheless it is conceivable that local tumor burden relative to local effector-to-target ratios vary and therefore different forms of B-cell malignancies may be eliminated by different effector mechanisms. For instance, rituximab appears to work predominantly via ADCC in both follicular lymphoma and chronic lymphocytic leukemia.[15,51] Differences in tumor burden or in local effector-to-target ratios may explain some of these observations. It is possible that the mechanism of action of CD20 monoclonal antobodies differ in initial treatment of advanced stage follicular non-Hodgkin's lymphoma from low grade follicular lymphoma with low tumor burden.

We have shown that a 10-fold difference in local tumor burden impacts the *in vivo* mechanism of action of CD20 monoclonal antibodies. Low tumor burden can be eliminated by a single effector mechanism, whereas high tumor burden requires the presence of multiple effector mechanisms. This finding could help us to better understand the *in vivo* mechanism of action of CD20 monoclonal antibodies.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat Rev Immunol. 2006;6 (5):394-403.
2. Lim SH, Beers SA, French RR, Johnson PW, Glennie MJ, Cragg MS. Anti-CD20 monoclonal antibodies: historical and future perspectives. Haematologica. 2010;95(1):135-43.
3. Glennie MJ, French RR, Cragg MS, Taylor RP. Mechanisms of killing by anti-CD20 monoclonal antibodies. Mol Immunol. 2007;44(16):3823-37.
4. Teeling JL, French RR, Cragg MS, van den BJ, Pluyter M, Huang H, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood. 2004;104(6):1793-800.
5. Teeling JL, Mackus WJ, Wiegman LJ, van den Brakel JH, Beers SA, French RR, et al. The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. J Immunol. 2006;177(1): 362-71.
6. Pawluczkowycz AW, Beurskens FJ, Beum PV, Lindorfer MA, van de Winkel JG, Parren PW, et al. Binding of submaximal C1q promotes complement-dependent cytotoxicity (CDC) of B cells opsonized with anti-CD20 mAbs ofatumumab (OFA) or rituximab (RTX): considerably higher levels of CDC are induced by OFA than by RTX. J Immunol. 2009;183(1):749-58.
7. Coiffier B, Lepretre S, Pedersen LM, Gadeberg O, Fredriksen H, van Oers MH, et al. Safety and efficacy of ofatumumab, a fully human monoclonal anti-CD20 antibody, in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: a phase 1-2 study. Blood. 2008;111(3):1094-100.
8. Osterborg A, Kipps TJ, Mayer J, Stilgenbauer S, Williams CD, Hellmen A, et al. Ofatumumab (HuMax-CD20), a novel CD20 monoclonal antibody, is an active treatment for patients with CLL refractory to both fludarabine and alemtuzumab or bulky fludarabine-refractory disease: results from the planned interim analysis of an international pivotal trial. Abstract #328.Presented at the American Society of Hematology Annual Meeting. 2008.
9. Beers SA, Chan CH, James S, French RR, Attfield KE, Brennan CM, et al. Type II (tositumomab) anti-CD20 monoclonal antibody out performs type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. Blood. 2008;112 (10):4170-7.
10. Byrd JC, Kitada S, Flinn IW, Aron JL, Pearson M, Lucas D, et al. The mechanism of tumor cell clearance by rituximab in vivo in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. Blood. 2002;99(3): 1038-43.
11. Ivanov A, Beers SA, Walshe CA, Honeychurch J, Alduaij W, Cox KL, et al. Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. J Clin Invest. 2009;119(8):2143-59.
12. Shan D, Ledbetter JA, Press OW. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood. 1998;91(5):1644-52.
13. de Haij S, Jansen JH, Boross P, Beurskens FJ, Bakema JE, Bos DL, et al. In vivo cytotoxicity of type I CD20 antibodies critically depends on Fc receptor ITAM signaling. Cancer Res. 2010;70(8):3209-17.
14. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med. 2000;6(4):443-6.
15. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood. 2002;99(3):754-8.
16. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol. 2003;21(21):3940-7.
17. Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, et al. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. J Exp Med. 2004;199(12):1659-69.
18. Hamaguchi Y, Xiu Y, Komura K, Nimmerjahn F, Tedder TF. Antibody isotype-specific engagement of Fcgamma receptors regulates B lymphocyte depletion during CD20 immunotherapy. J Exp Med. 2006;203(3):743-53.
19. Nimmerjahn F, Ravetch JV. Antibodies, Fc receptors and cancer. Curr Opin Immunol. 2007;19(2):239-45.
20. Anolik JH, Campbell D, Felgar RE, Young F, Sanz I, Rosenblatt J, et al. The relationship of FcgammaRIIIa genotype to degree of B cell depletion by rituximab in the treatment of systemic lupus erythematosus. Arthritis Rheum. 2003;48(2):455-9.
21. Gong Q, Ou Q, Ye S, Lee WP, Cornelius J, Diehl L, et al. Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. J Immunol. 2005;174(2):817-26.
22. Di Gaetano N., Cittera E, Nota R, Vecchi A, Grieco V, Scanziani E, et al. Complement activation determines the therapeutic activity of rituximab in vivo. J Immunol. 2003; 171(3):1581-7.
23. Golay J, Cittera E, Di GN, Manganini M, Mosca M, Nebuloni M, et al. The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes. Haematologica. 2006;91(2):176-83.
24. Kennedy AD, Beum PV, Solga MD, DiLillo DJ, Lindorfer MA, Hess CE, et al. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. J Immunol. 2004;172(5):3280-8.
25. Wang SY, Racila E, Taylor RP, Weiner GJ. NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement. Blood. 2008; 111(3):1456-63.
26. van Meerten T, van Rijn RS, Hol S, Hagenbeek A, Ebeling SB. Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity. Clin Cancer Res. 2006;12(13): 4027-35.
27. Beers SA, French RR, Chan HT, Lim SH,

Jarrett TC, Vidal RM, et al. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. Blood. 2010;115(25):5191-201.
28. Berinstein NL, Grillo-Lopez AJ, White CA, ce-Bruckler I, Maloney D, Czuczman M, et al. Association of serum rituximab (IDEC-C2B8) concentration and anti-tumor response in the treatment of recurrent low-grade or follicular non-Hodgkin's lymphoma. Ann Oncol. 1998;9(9):995-1001.
29. Dayde D, Ternant D, Ohresser M, Lerondel S, Pesnel S, Watier H, et al. Tumor burden influences exposure and response to rituximab: pharmacokinetic-pharmacodynamic modeling using a syngeneic bioluminescent murine model expressing human CD20. Blood. 2009;113(16):3765-72.
30. Bleeker WK, Munk ME, Mackus WJ, van den Brakel JH, Pluyter M, Glennie MJ, et al. Estimation of dose requirements for sustained in vivo activity of a therapeutic human anti-CD20 antibody. Br J Haematol. 2008;140(3):303-12.
31. Bevaart L, van Ojik HH, Sun AW, Sulahian TH, Leusen JH, Weiner GJ, et al. CpG oligodeoxynucleotides enhance FcgammaRI-mediated cross presentation by dendritic cells. Int Immunol. 2004;16(8): 1091-8.
32. Lachmann PJ. Preparing serum for functional complement assays. J Immunol Methods. 2010;352(1-2):195-7.
33. Klerx JP, Beukelman CJ, van DH, Willers JM. Microassay for colorimetric estimation of complement activity in guinea pig, human and mouse serum. J Immunol Methods. 1983;63(2):215-20.
34. Beurskens FJ, Ruuls SR, Engelberts PJ, Vink T, Mackus WJ, van de Winkel JG, et al. Complement activation impacts B-cell depletion by both type I and type II CD20 monoclonal antibodies. Blood. 2008;112 (10):4354-5.
35. Yazawa N, Hamaguchi Y, Poe JC, Tedder TF. Immunotherapy using unconjugated CD19 monoclonal antibodies in animal models for B lymphocyte malignancies and autoimmune disease. Proc Natl Acad Sci USA. 2005;102(42):15178-83.
36. Lefebvre ML, Krause SW, Salcedo M, Nardin A. Ex vivo-activated human macrophages kill chronic lymphocytic leukemia cells in the presence of rituximab: mechanism of antibody-dependent cellular cytotoxicity and impact of human serum. J Immunother. 2006;29(4):388-97.
37. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol. 2008;8(1):34-47.
38. Schmidt RE, Gessner JE. Fc receptors and their interaction with complement in autoimmunity. Immunol Lett. 2005;100(1): 56-67.
39. Bevaart L, Jansen MJ, van Vugt MJ, Verbeek JS, van de Winkel JG, Leusen JH. The high-affinity IgG receptor, FcgammaRI, plays a central role in antibody therapy of experimental melanoma. Cancer Res. 2006;66(3): 1261-4.
40. Heel KA, Hall JC. Peritoneal defences and peritoneum-associated lymphoid tissue. Br J Surg. 1996;83(8):1031-6.
41. Abe Y, Kuroda Y, Kuboki N, Matsushita M, Yokoyama N, Kojima N. Contribution of complement component C3 and complement receptor type 3 to carbohydrate-dependent uptake of oligomannose-coated liposomes by peritoneal macrophages. J Biochem. 2008;144(5):563-70.
42. Minard-Colin V, Xiu Y, Poe JC, Horikawa M, Magro CM, Hamaguchi Y, et al. Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage FcgammaRI, FcgammaRIII, and FcgammaRIV. Blood. 2008;112(4):1205-13.
43. van Spriel AB, Leusen JH, van EM, Dijkman HB, Assmann KJ, Mayadas TN, et al. Mac-1 (CD11b/CD18) is essential for Fc receptor-mediated neutrophil cytotoxicity and immunologic synapse formation. Blood. 2001;97(8):2478-86.
44. van Spriel AB, van Ojik HH, Bakker A, Jansen MJ, van de Winkel JG. Mac-1 (CD11b/CD18) is crucial for effective Fc receptor-mediated immunity to melanoma. Blood. 2003;101(1):253-8.
45. Imai M, Landen C, Ohta R, Cheung NK, Tomlinson S. Complement-mediated mechanisms in anti-GD2 monoclonal antibody therapy of murine metastatic cancer. Cancer Res. 2005;65(22):10562-8.
46. Taylor RP, Lindorfer MA. Immunotherapeutic mechanisms of anti-CD20 monoclonal antibodies. Curr Opin Immunol. 2008;20(4):444-9.
47. Taylor RP. Use of fresh frozen plasma to enhance the therapeutic action of rituximab. QJM. 2008;101(12):991-2.
48. Williams ME, Densmore JJ, Pawluczkowycz AW, Beum PV, Kennedy AD, Lindorfer MA, et al. Thrice-weekly low-dose rituximab decreases CD20 loss via shaving and promotes enhanced targeting in chronic lymphocytic leukemia. J Immunol. 2006;177(10) :7435-43.
49. Wang SY, Veeramani S, Racila E, Cagley J, Fritzinger DC, Vogel CW, et al. Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model. Blood. 2009;114(26):5322-30.
50. Kennedy AD, Solga MD, Schuman TA, Chi AW, Lindorfer MA, Sutherland WM, et al. An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20+ cells by rituximab. Blood. 2003; 101(3):1071-9.
51. Zent CS, Secreto CR, Laplant BR, Bone ND, Call TG, Shanafelt TD, et al. Direct and complement dependent cytotoxicity in CLL cells from patients with high-risk early-intermediate stage chronic lymphocytic leukemia (CLL) treated with alemtuzumab and rituximab. Leuk Res. 2008;32(12): 1849-56.