Microenvironment and anti-CD20 based therapies in CLL
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CD40 stimulation in vivo does not affect anti-CD20 mediated B cell depletion

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Submitted
Abstract

The mechanism responsible for resistance to anti-CD20 mediated therapy in the treatment of B cell malignancies is poorly understood. Since CD40-CD40L interactions provide important survival signals to B cells, we tested the effect of CD40 stimulation on the susceptibility of B cells to both type I (rituximab) and type II (GA101) anti-CD20 mAbs in human CD20 transgenic (hCD20TG) mice. We found that CD40 stimulation in vitro sensitized hCD20TG B cells to cell death induced by either rituximab or GA101. CD40 triggering in vivo induced a marked increase in B cell numbers, but did not affect anti-CD20 induced depletion of B cell subsets in either blood, spleen or lymph nodes. Anti-CD40 pre-treatment did protect pro- and mature B cells in the bone marrow from anti-CD20 mediated depletion. No differences were observed between Rituximab- or GA101-induced B cell depletion. Altogether, we conclude that for most B cell subsets CD40-CD40L interaction is an unlikely mechanism of resistance to anti-CD20 monoclonal antibodies in vivo.
Introduction

The introduction of rituximab, a chimeric anti-CD20 mAb, has considerably improved the outcome for patients with B cell malignancies. However, a substantial proportion of patients are resistant or acquire rituximab resistance following therapy 1-3. Therefore new and more potent anti-CD20 mAbs are continuously being sought. Currently, two different types of anti-CD20 mAbs are available, namely type I (e.g. rituximab, ofatumumab) and type II (e.g. tositumomab, GA101), which differ in their ability to redistribute CD20 into lipid rafts and in their potency to induce complement dependent cytotoxicity (CDC), homotypic aggregation and programmed cell death (PCD) 4;5.

The underlying mechanisms of resistance to anti-CD20 mediated therapy are poorly understood. Internalization of CD20 by B cells leading to reduced antibody-dependent cellular cytotoxicity (ADCC) and to degradation of CD20:mAb complexes 6, cell intrinsic resistance to rituximab 7 and micro-environmental factors and circulatory dynamics of B cells 8 are thought to play a role. An important micro-environment-derived survival signal for B cells is CD40-triggering. CD40 stimulation can be provided by interaction with CD40L on CD4+ T cells 9;10. Previous studies have shown that CD40 stimulation increases the anti-apoptotic profile of CLL cells (11,12), resulting in drug resistance 11;12. In sharp contrast however, CD40 stimulation of CLL cells sensitizes to anti-CD20 mediated cell death in vitro 13;14. Although these findings could lead to new strategies that can boost anti-CD20 mediated therapy, it is not yet known whether CD40 triggering also increases the susceptibility of B cells to anti-CD20 mAbs in vivo. Therefore, we investigated anti-CD20 mediated B cell depletion in hCD20 TG mice that were pre-treated or not with anti-CD40 mAbs. Since the most important mechanism of depletion differs between type I- and type II anti-CD20 mAbs (CDC and ADCC versus direct cell death, respectively 15) the effect of CD40 stimulation on sensitivity for type I- (rituximab) and type II- (GA101) 16 anti-CD20 mAbs was tested separately. In vitro, CD40 stimulation sensitized for both rituximab and GA101 induced cell death. However, in vivo triggering through CD40 did not affect anti-CD20 induced B cell depletion, with the exception for particular B cell subsets in the bone marrow. These data reveal important differences between in vitro and in vivo effects of CD40-triggering on the susceptibility to anti-CD20 antibodies. Since CD40-stimulation in vivo induces strong B cell proliferation, our findings argue for caution in the application of CD40 triggering for treatment of B cell malignancies.

Methods

Mice, anti-CD40- and anti-CD20 treatment

Human CD20TG mice on a balb/c background have been described previously 17 and were kindly provided by Dr. Beers (University of Southampton, Southampton, UK). Wild-type
balb/c mice were obtained from Jackson Laboratories (Maine, USA). Mice were bred and maintained in local facilities under specific pathogen-free conditions. On day -2 and 0, mice were pre-treated with 100 µg rat anti-mouse anti-CD40 mononclonal antibody (FGK-45) (Bioceros, Utrecht, The Netherlands) or PBS i.p. On day 1 mice were treated with 0.5 mg rituximab (Roche) or GA101 (Roche/Glycart, Switzerland) or PBS i.p. On day 5 mice were sacrificed and blood and lymphoid organs were analyzed by flow cytometry. All animal experiments were approved by the Animal Care and Use Committee of the Academic Medical Center, Amsterdam. Approval ID of this study: DSK100978.

Cell preparation, antibodies and flow cytometry
Single cell suspensions were prepared from spleens, mesenteric lymph nodes and bone marrow (1 femur). Erythrocytes were lysed with ammonium chloride buffer. After isolation, single cell suspensions were stained with different mAb conjugates. FACS antibodies were purchased from Beckton Dickinson (San Jose, CA), Beckman Coulter (Woerden, The Netherlands) and eBioscience (San Diego, CA). For intracellular Ki-67 expression analysis, cells were fixed and permeabilised (eBioscience, San Diego, CA) and subsequently stained with FITC-conjugated Ki-67 or isotype control (Becton Dickinson, San Jose, CA). Unspecific binding of mAbs was blocked by adding 1.25 µg/ml anti-CD16/32 (clone 2.4G2 Bioceros, Utrecht, The Netherlands)

Expression of cell surface molecules and intracellular proteins was determined using the FACSCalibur or FACSanto flow cytometer (BD biosciences) and CellQuest software (Beckton Dickinson) was used for data acquisition. Data were analyzed with FlowJo software (TreeStar, San Carlos, CA, USA).

CD40 stimulation of splenocytes and induction of cell death with anti-CD20 mAbs in vitro
After isolation cells were cultured in round-bottom 96 well plates in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml gentamycin and 0.00036% b-mercaptoethanol. Cells were stimulated with 5 µg/ml rat anti-mouse anti-CD40 monoclonal antibody (FGK-45) (Bioceros, Utrecht, The Netherlands), 5 µg/ml anti-IgM (HB88) or both. After 48 hours, cells were incubated with 10 µg/ml rituximab, 10 µg/ml GA101 in the presence or absence of a secondary goat-anti-human Fc-g specific crosslinking mAb (Jackson Immunoresearch Europe). Cell death was analyzed after a subsequent incubation period of 24 hours, by evaluation of mitochondrial membrane potential with MitoTracker orange (Molecular probes, Leiden, The Netherlands) according to the manufacturer’s recommendations. Data are expressed as specific cell death: % cell death in stimulated cells - % cell death in medium control.
Statistics and calculation of relative depletion

Assuming Gaussian distribution, a two-sided unpaired t-test was used to analyze differences between the groups. P-values < 0.05 were considered to be statistically significant.

To calculate anti-CD20 induced relative B cell depletion in PBS-or anti-CD40 pre-treated mice, the mean B cell count was calculated from 3 mice in the PBS or anti-CD40 treated group and set as 100%. Relative depletion in Rituximab or GA101 treated mice was calculated as follows: 100 – (B cell count rituximab or GA101 treated mice/mean B cell count PBS mice*100).

Relative depletion in CD40+Rituximab or CD40+GA101 treated mice was calculated as follows: 100 – (B cell count CD40+rituximab or CD40+GA101 mice/mean B cell count CD40 mice*100).

Results

CD40 stimulation of hCD20TG splenocytes sensitizes to anti-CD20 mediated cell death in vitro

To test whether CD40 stimulation influenced susceptibility to anti-CD20 monoclonal antibodies, freshly isolated splenocytes from hCD20TG mice were stimulated for 48 hours with agonistic anti-CD40 mAb (FGK-45) and/or anti-IgM (HB88), after which the cells were incubated with rituximab or GA101 in the presence or absence of a crosslinking mAb (XL). We found that CD40 stimulation increased anti-CD20 induced cell death, whereas BCR triggering alone did not sensitize to anti-CD20 induced cell death but protected cells from apoptosis (Figure 1). The combination of BCR- and CD40 triggering showed no further

![Figure 1. CD40 stimulation of hCD20TG splenocytes sensitizes to anti-CD20 mediated cell death in vitro. Single cell suspensions were isolated from fresh hCD20TG spleens and stimulated with anti-CD40, anti-IgM or both. After 48 hours cells were incubated with 10 μg/ml rituximab or GA101 in the presence or absence of 50μg/ml crosslinking antibody (XL). After 24 hours cell death was analyzed by evaluation of mitochondrial membrane potential with MitoTracker orange in B220+ cells. CD40 stimulation increased anti-CD20 induced cell death, whereas BCR triggering alone did not sensitize to anti-CD20 induced cell death but protected cells from apoptosis. Combination of BCR- and CD40 triggering showed no further increase in sensitization than CD40 triggering alone. Averaged results from 2 mice are presented as percentage specific cell death (mean ± SEM).](image-url)
increase in sensitization than CD40 triggering alone, indicating a CD40 specific effect. In contrast to GA101, Rituximab induced apoptosis only in the presence of XL14, indicating that GA101 is more efficient in direct cell death induction in vitro than rituximab. No cell death was observed in CD40-stimulated splenocytes of non-transgenic mice upon incubation with anti-CD20 mAbs (data not shown).

**CD40 stimulation in vivo induces B cell depletion in blood and increases B cell numbers in lymphoid organs by inducing proliferation**

To assess the role of CD40-stimulation in putative anti-CD20 mAb resistance in vivo, hCD20TG mice were injected twice with anti-CD40 mAb (on day -2 and 0). Five days after the last injection, an upregulation of MHC class II was observed on B cells (data not shown). As expected 18,19, CD40 treatment induced splenomegaly with a marked increase in B cell numbers (Figure 2A). Also in lymph nodes, B cell numbers were increased after CD40 treatment (Figure 2A). In bone marrow, the number of total B220+ cells did not change (Figure 2A), however the subpopulation of B220+CD20+ cells increased after CD40 treatment (Figure 2A). In contrast to lymphoid organs, a profound B cell depletion in blood was observed after CD40 treatment (Figure 2A), which is most likely due to redistribution of B cells. Although we cannot completely exclude CD40-induced apoptosis of mature, circulating B cells as described by Rathmell et al, we consider this unlikely as B cell numbers in the secondary lymphoid organs increased 20. To test whether increased B cell numbers in lymphoid organs upon anti-CD40 treatment was due to increased proliferation, Ki-67 expression was analyzed in splenocytes. Figure 2C shows a significant increase in Ki-67+ B cell (B220+) numbers after CD40 treatment. As expected, no change in numbers or Ki-67 expression in T cells (CD3+) was observed in anti-CD40 treated mice (Figure 2B and 2C).

**CD40 stimulation in vivo neither sensitizes nor protects against anti-CD20 mediated cell death**

To assess whether CD40 stimulation also sensitizes B cells to anti-CD20 mediated cell death in vivo, hCD20TG mice (and WT mice as control) were injected with anti-CD40 mAb or PBS (on day -2 and 0) and subsequently treated with rituximab or GA101 (on day 1). Five days following anti-CD20 treatment, mice were sacrificed and blood and lymphoid organs were analyzed. Both rituximab and GA101 induced massive systemic B cell depletion, which still occurred when mice were pretreated with anti-CD40 mAb (Figure 3A). Relative depletion in all groups was comparable, as shown in Figure 3B, indicating that CD40 stimulation in vivo does neither sensitize nor protect against anti-CD20 induced B cell depletion. A significant increase in Ki-67+ B cell (B220+) numbers was observed after CD40 treatment (Figure 2C+ Figure 3C) and interestingly anti-CD20 mAbs preferentially depleted CD40-induced proliferating cells as shown in Figure 3C. WT mice treated with anti-CD20 mAbs showed no B cell depletion, also not after pre-treatment with anti-CD40 (data not shown).
CD40 stimulation in vivo does not affect anti-CD20 mediated B cell depletion

Figure 2. CD40 stimulation in vivo induces B cell depletion in blood and increases B cell numbers in lymphoid organs by inducing proliferation.

A. Total B cell numbers (#B220$^+$ cells) in Blood, Sp, LN, BM in mice after CD40 treatment. After CD40 treatment a profound decrease in B cells was observed in blood and increased B cell numbers (B220$^+$CD20$^+$) were found in Sp, LN and BM. In bone marrow the number of total B220$^+$ cells and the subpopulation of B220$^+$CD20$^+$ cells is shown. Mice were sacrificed five days after the last anti-CD40 injection. Averaged results from 3 mice/group are presented as total number (#) B220$^+$ cells (mean± SEM). 0.01<p<0.05 *, 0.001<p<0.01 **, p<0.001 ***

B. No difference in total T cell numbers (#CD3$^+$ cells) in Blood, Sp, LN, BM in mice after CD40 treatment. Mice were sacrificed five days after the last anti-CD40 injection. Averaged results from 3 mice/group are presented as total number (#) CD3$^+$ cells (mean± SEM).

C. Intracellular Ki-67 staining of splenocytes (B cells: B220$^+$ T cells: CD3$^+$) from mice treated with anti-CD40 or PBS. CD40 stimulation induced an increase in Ki-67$^+$ B cells. No increase in Ki-67$^+$ T cells was found after CD40 stimulation. Mice were sacrificed five days after the last anti-CD40 injection. Averaged results from 3 mice/group are presented as percentage or number Ki-67$^+$ cells (mean± SEM). 0.01<p<0.05 *
Figure 3. CD40 stimulation in vivo neither sensitizes nor protects against anti-CD20 mediated cell death.

A. upper panel: representative FACS plots of B220+ and CD3+ splenocytes. Lower panel: total number of B220+ cells in Blood, Sp, LN and BM after CD40 treatment, CD20 treatment (GA101 (GA) or Rituximab (Rit)) or the combination. Mice were sacrificed 5 days following CD20 treatment. White bars indicate control, black bars indicate CD40 treated mice. Both rituximab and GA101 induced massive systemic B cell depletion, still observed after pretreatment with anti-CD40 mAb. Averaged results from 3 mice/group are presented as total number (#) B220+ cells (mean± SEM). .01<p< .05 *

B. Relative depletion of total B220+ cells is comparable in Blood, Sp, LN and BM 5 days after GA101 or Rituximab treatment in CD40 treated or PBS treated mice. White bars indicate control, black bars indicate CD40 treated mice.

C. Intracellular Ki-67 staining of splenocytes (B cells: B220+ T cells: CD3+) from mice treated with anti-CD40, anti-CD20 or both. After CD40 treatment, a significant increase in Ki-67+ B cell (B220+) numbers was observed and anti-CD20 mAbs preferentially depleted CD40-induced proliferating cells. White bars indicate control, black bars indicate CD40 treated mice. Averaged results from 3 mice/group are presented as number Ki-67+ cells (mean± SEM). .01<p< .05 *
CD40 stimulation does not affect anti-CD20 mediated depletion of B cell subsets in spleen, but protects pro- and mature B cells in bone marrow

As we found no difference of CD40-treatment on CD20-mediated depletion of the total pool of B cells (B220+ cells), we tested whether particular B cell subsets in spleen and bone marrow were differentially affected by this treatment. First, we examined whether anti-CD20 altered the expression of CD20 on these cells.

Figure 4A shows CD20 expression levels on different B cell subsets in the spleen after CD40 treatment. CD40 stimulation did not influence expression of CD20, except on plasmacells (PCs) (B220-CD138-CD38+), where CD20 was downregulated after CD40 stimulation. In humans, PCs have no CD20 expression 21,22 and are therefore resistant to anti-CD20 induced cell death 23. In hCD20TG mice, PCs and plasmablasts (Plbs) expressed CD20 (Figure 4A). Interestingly, B cells with a germinal center (GC) B cell phenotype (B220+CD38-GL-7+) expressed very high levels of CD20 (Figure 4A) and we found that the absolute number of these cells increased significantly upon CD40 stimulation (Figure 4B). Correspondingly, CD40-induced GC cells were sensitive to both rituximab and GA101 (Figure 4B). In the lymph nodes the same findings were observed (not shown).

An increase in PCs (B220-CD138+CD38+) and Plbs (B220+CD138+CD38++) was also observed after CD40 treatment (Figure 4B). However, no significant decrease was observed in PCs numbers in all groups after anti-CD20 therapy, despite CD20 expression albeit low (Figure 4A). Furthermore, CD40 stimulation increased the number of mature (B220+CD23+,IgM+,CD21/35+), T2 (B220+CD23+,IgM+,CD21/35++), MZB (B220+CD23+,IgM+,CD21/35+) and T1 (B220+CD23-,IgM+,CD21/35+) B cells (Figure 4B), which were all strongly depleted after anti-CD20 therapy (Figure 4B). No differences between GA101 and rituximab were found.

After CD40 stimulation, CD20 expression remained unchanged in pre- and immature B cells in bone marrow and showed a slight increase on pro- and mature B cells (Figure 4C). Figure 4D shows depletion of pro-B cells (B220+CD43b+) and mature B cells (B220hiCD43b-) in bone marrow of non-CD40 pretreated mice. However, CD40 treatment protected these cells from anti-CD20 mediated depletion (Figure 4D). In line with previous studies 8, pre-B cells (B220+CD43b-IgM-) and immature B cells (B220+CD43b+IgM+) were resistant to anti-CD20 mAbs (Figure 4D). The number of pre-B cells even increased after anti-CD20 treatment (Figure 4D). In conclusion, these results indicate that CD40 stimulation protects pro- and mature B cells in bone marrow and has no effect on CD20-mediated depletion of B cell subsets in the spleen.

Discussion

In this study we show that CD40 stimulation in vitro sensitizes hCD20TG splenocytes to anti-CD20 induced cell death, confirming earlier findings in CLL13,14. Interestingly, CD40
Figure 4. CD40 stimulation does not affect anti-CD20 mediated depletion of B cell subsets in spleen, but protects pro- and mature B cells in bone marrow despite an increase in CD20 expression

A. Representative histograms of CD20 expression on different B cell subsets in spleen analyzed by flow cytometry, shaded: WT, grey: hCD20TG PBS, black: hCD20TG after CD40 treatment. Right: mean fluorescence intensity (MFI) of CD20 on different B cell subsets from spleen after CD40 treatment. White bars indicate control, black bars indicate CD40 treated mice. CD40 stimulation did not influence expression of CD20, except on plasmacells (PCs) (B220-CD138+CD38+) where CD20 was downregulated after CD40 stimulation. Averaged results from 3 mice/group are presented as MFI of CD20 (mean ± SEM). Mat=mature B cells, MZB=marginal zone B cells, PC=plasmacells, Plb=plasmablasts

B. Bar graphs representing total number of cells (mean± SEM of 3 mice/group) of B220+ splenocytes stained for germinal center (GC) cells (B220+CD38-GL-7-), plasmacells (PCs) (B220+CD138+CD38-) and (plasmablasts) Plbs (B220+CD138+CD38+++) and mature (B220+CD23-,IgM+,CD21/35+), T2 (B220+CD23-,IgM+,CD21/35++) and marginal zone B cells (MZB) (B220+CD23-,IgM+,CD21/35++) and T1 (B220+CD23+,IgM+,CD21/35+) B cells after treatment with anti-CD40, anti-CD20 or both. White bars indicate control, black bars indicate CD40 treated mice. After CD40 treatment an increase in GC, PC, Plb, mature, T2, MZB and T1 cells was observed. All B cell subsets were depleted after CD20 therapy, except GC cells. No difference between rituximab and GA101 was found. Mice were sacrificed 5 days following
CD40 stimulation in vivo does not affect anti-CD20 mediated B cell depletion.

CD20 treatment. 01<p<.05 *, .001<p<.01 **.

C. Representative histograms of CD20 expression on different B cell subsets from BM analyzed by flow cytometry shaded: WT, grey: hCD20TG PBS, black: hCD20TG after CD40 treatment. Right: mean fluorescence intensity (MFI) of CD20 on different B cell subsets from BM after CD40 treatment. CD20 expression showed a slight increase on pro- and mature B cells. White bars indicate control, black bars indicate CD40 treated mice. Averaged results from 3 mice/group are presented as MFI of CD20 (mean ± SEM). Pro=pro B cells, pre=pre B cells, Imm=immature B cells, mat=mature B cells

D. Total number of pro B cells (B220^-CD43b^+), pre B cells (B220^-CD43b^+IgM^-), immature B cells (B220^+CD43b^-IgM^-) and mature B cells (B220^+CD43b^-IgM^-) after CD40 treatment, CD20 treatment or the combination. White bars indicate control, black bars indicate CD40 treated mice. CD40 treatment protected pro- and mature B cells from anti-CD20 mediated depletion. Pre B cells (B220^-CD43b^+IgM^-) and immature B cells (B220^-CD43b^+IgM^-) were resistant to anti-CD20 mAbs. Mice were sacrificed 5 days following CD20 treatment. Averaged results from 3 mice/group are presented as number (#) cells (mean± SEM). .01<p<.05 *, .001<p<.01 **, p<.001 ***

stimulation in vivo does not sensitize B cells to anti-CD20 mediated depletion. Actually, CD40 stimulation results in splenomegaly with a strong increase in B cell numbers and proliferation as described earlier 18;19;24. These findings therefore argue for caution in the application of CD40 triggering for treatment of B cell malignancies as suggested by some researchers 25;26. Anti-CD40-induced splenomegaly is probably attributable to effects on multiple cell types 19 and is partly related to non-direct effects of CD40 stimulation e.g. changes in red pulp cell types, cytokine secretion, increase in stromal cells and dendritic cells (DCs) 19. This study clearly shows CD40-induced increase in Ki-67+ B cells in the spleen. Despite the fact that CD40-stimulation strongly increased the susceptibility of B cells for anti-CD20 induced cell death in vitro, it did not do so in vivo. Given the high sensitivity of normal B cells to CD20 mediated B cell depletion however, detecting sensitization may be difficult in this system. Furthermore, the in vivo model might represent a complex phenotype since CD40 stimulation induced proliferation of B cells on one hand but on the other hand may have sensitized B cells to anti-CD20 treatment at the same time. Moreover, it should be stressed that in our model we could only study depletion of normal B cells and their response to CD40 potentiation may differ from those of malignant B cell clones. Using the current treatment regimen, we found no differences between rituximab or GA101 induced B cell depletion, also not after CD40 stimulation. This is intriguing, since the mechanism of depletion differs between these types of antibodies. However, it could be that changes in the treatment protocol or a longer follow up time might reveal differences between these different anti-CD20 mAbs.

Although we found no difference of CD40-treatment on CD20-mediated depletion of the total pool of B cells (B220^+ cells) our data suggest, that CD40 stimulation in vivo protects pro- and mature B cells against anti-CD20 mediated depletion, despite an increase in CD20 expression levels. The protective effect on pro B cells is most likely an indirect effect of CD40 stimulation as CD40 is undetectable at the pro-B cell stage 9;27. Another possible explanation is that anti-CD20 treatment promoted pro-B-to-pre-B cell transition, which was blocked by anti-CD40 treatment via an unknown mechanism. This hypothesis is supported by the interesting finding that the number of pre B cells appeared to increase after CD20 treatment.
in non-CD40 treated mice. Whether this is the result of increased differentiation of pro B cells into pre B cells or increased proliferation of pre B cells is yet unclear. Obviously, these analyses are based on the assumption that expression of the cell surface markers used for the identification of these individual subsets is not changed by the anti-CD20 therapy itself. The fact that precursor cells in the bone marrow are protected against anti-CD20 mediated depletion after CD40 stimulation may have important implications in possible therapeutic use of CD40 ligation in vivo, where reconstitution of B cells after CD20 therapy will be expected earlier and faster. The fact that mature B cells in the bone marrow are protected from CD20 induced depletion by CD40 ligation, but mature B cells in the spleen and LN are not, could be explained by different micro-environmental factors. Gong et al hypothesize that resistance of GC cells to anti-CD20 mAbs is caused by CD40-CD40L interaction. However, here we show for the first time that CD40 stimulation in vivo does not influence susceptibility of GC cells to anti-CD20 mAbs. Previous studies showed resistance of MZB cells to anti-CD20 therapy and the important role of BAFF herein. In contrast, our study shows that CD40-induced-MZB cells are sensitive to both rituximab and GA101 induced cell death. Finally, in Gong’s study, treatment with anti-CD20 mAb incompletely depleted splenic B cells remaining almost 30% of the B cells, whereas depletion is approximately 90% in our study (Figure 3B). Our findings are based on a single dose of anti-CD40 (based on a half-life of 2-3 days) and anti-CD20. Obviously, different regimens, e.g. variation in strength of CD40 stimulation, dose of anti-CD20 mAb and subsequent follow up of B cell depletion in time could give different results. The resistance of CD20 expressing PCs and plasmablasts to depletion is interesting. DiLillo et al showed that PCs in vivo express low but detectable levels of mouse CD20 and that long-lived and short-lived PC numbers were unchanged after anti-CD20 treatment. These findings suggests cell intrinsic resistance, but the underlying mechanism has yet to be uncovered.

Based on our in vitro data with CLL cells and hCD20TG splenocytes that show CD40-induced sensitization to anti-CD20 induced cell death, an in vivo approach combining anti-CD40- and anti-CD20 mAbs could be promising in the treatment of (rituximab resistant) B cell malignancies. Interestingly, CD40 mAb can activate both neoplastic CD40+ B cells and host antigen presenting cells to present endogenous antigen or cross prime exogenous antigen respectively from necrotic or apoptotic tumor, which will eventually lead to anti-tumor cytotoxic T cell formation. By inducing cell death in neoplastic B cells, anti-CD20 mAbs will likely increase anti-CD40-induced antigen presentation. However, this study shows that in vivo treatment with CD40 mAbs should be approached with caution. CD40 triggering in vivo results in a profound increase in B cell numbers, which could be detrimental in patients with B cell malignancies. On the other hand, the present study shows that the majority of CD40-induced B cell subsets are depleted with anti-CD20 mAbs. Furthermore, these data suggest that CD40-CD40L interactions do not play a major role in anti-CD20 resistance in vivo. Yet, to further assess the role of CD40 triggering on susceptibility of malignant B cells to anti-CD20 mAbs in vivo, ‘CLL-like’ or lymphoma mouse models should be tested.
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Reference List


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