CD20 up-regulation in pediatric B-cell precursor acute lymphoblastic leukemia during induction treatment: setting the stage for anti-CD20 directed immuno-therapy

Michael N. Dworzak¹, Angela Schumich¹, Dieter Printz¹, Ulrike Pötschger¹, Zvenyslava Husak¹, Andishe Attarbaschi², Giuseppe Basso³, Giuseppe Gaipa⁴, Richard Ratei⁵, Georg Mann², and Helmut Gadner¹,², for the AIEOP-BFM-ALL-FCM-MRD-Study Group

¹Children’s Cancer Research Institute, Vienna, Austria
²St. Anna Children’s Hospital, Vienna, Austria
³Laboratory of Pediatric Onco-Hematology, Department of Pediatrics, University Hospital of Padova, Padova, Italy
⁴Tettamanti Research Center, Department of Pediatrics, University of Milano-Bicocca, Ospedale San Gerardo, Monza, Italy
⁵Dept. of Hematology, Oncology and Tumor Immunology, Robert-Roessle-Clinic at the HELIOS Klinikum Berlin, Charité Medical School, Berlin, Germany

Running short title: CD20 up-regulation in childhood ALL during induction

Correspondence: MN Dworzak, M.D., Associate Professor of Pediatrics
Children’s Cancer Research Institute (CCRI) and St. Anna Kinderspital, Kinderspitalgasse 6, A-1090 Vienna, Austria
Tel.: +43-1-40170; Fax: +43-1-40470-7150; E-mail: dworzak@stanna.at
Abstract

CD20 is expressed in about one half of pediatric acute lymphoblastic leukemia (ALL) cases with B-cell precursor (BCP) origin. We observed recently that it is further up-regulated in some patients during induction treatment. To understand the impact of this on the potential effectiveness of anti-CD20 immunotherapy, we studied 237 CD10-positive pediatric BCP-ALL patients consecutively recruited to trial AIEOP-BFM-ALL-2000. We analyzed CD20 expression changes from diagnosis to end-induction, focusing on sample pairs with ≥0.1% residual leukemic blasts (MRD), and assessed complement-induced cytotoxicity by CD20-targeting with rituximab in-vitro (n=9 paired samples).

CD20-positivity significantly increased from 45% in initial samples to 81% at end-induction (day 15: 71%). Also the levels of expression increased, so that 52% of cases at end-induction showed ≥90% CD20pos leukemic cells, as opposed to 5% at diagnosis (day 15: 20%). CD20 up-regulation was frequent in high-risk patients, patients with high MRD at end-induction, and patients who suffered later from relapse, but not in TEL/AML1 cases. Notably, up-regulation occurred in viable cells sustaining chemotherapy. In-vitro, CD20 up-regulation significantly enhanced rituximab-cytotoxicity and could be elicited upon prednisolone incubation.

In summary, CD20 up-regulation is frequently induced in BCP-ALL during induction and this translates into an acquired state of higher sensitivity to rituximab. This study was registered at http://www.clinicaltrials.gov/ as NCT00430118.
Introduction

CD20 is a signature B-cell differentiation antigen strongly expressed on the surface of mature normal as well as malignant B-cells. It is also expressed, but at lower levels and with larger variance, on more immature B-cells and their malignant counterparts found in B-cell precursor (BCP) acute lymphoblastic leukemia (ALL).\(^1,2\) In line with the expression patterns anti-CD20 directed immunotherapy has been shown to elicit potent anti-tumor effects specifically in mature B-cell lymphoma and leukemia, where it has been incorporated into standard treatment as a valuable therapy advance.\(^3\) To date, the most broadly evaluated compound for CD20-targeting is rituximab, a chimeric antibody that was licensed by the FDA in 1997 as the first anticancer monoclonal antibody. It acts by complement-dependent and antibody-dependent cell-mediated cytotoxicity, as well as by inducing apoptosis directly.\(^4\) Recently, targeted therapy with rituximab has been implicated also in BCP-ALL for combination with conventional chemotherapy,\(^5\) with at least six active trials listed at [http://www.clinicaltrials.gov/](http://www.clinicaltrials.gov/) as per May 2008. In children with BCP-ALL, published usage has been confined mostly to anecdotal reports on relapsed or refractory disease.\(^6-9\) Importantly, activity can be anticipated primarily in CD20-positive cases which relevantly limits its applicability in pediatric BCP-ALL supposedly to less than one half of patients as determined at diagnosis.\(^2\)

During the course of an internationally collaborative study on flow cytometric minimal residual disease (MRD) assessment in childhood ALL, we noted that phenotypic modulation occurred regularly in viable leukemic cells resisting induction treatment with protocol AIEOP-BFM 2000.\(^10,11\) As one of the most frequently observed phenotypic changes, CD20 expression was found to be up-regulated. Gene expression analysis
showed that this increase in surface protein density is paralleled by up-regulation of mRNA expression as early as at day 8 after start of treatment. The phenomenon has largely been attributed to glucocorticoid action. We hypothesized that this increase in CD20 expression could be exploited for anti-CD20 targeted therapy, setting the stage that more patients with BCP-ALL than assessable upon phenotypic analysis at diagnosis could eventually profit from such treatment. To get a more comprehensive view of the frequency and the potential impact of CD20 up-regulation, we analyzed this in a large cohort of pediatric BCP-ALL patients recruited to a nation-wide treatment protocol in Austria and also assessed the effects of rituximab in paired in-vitro analyses.
Materials and methods

Patients, samples, and treatment

Between December 2000 and June 2006, 306 patients with ALL (age 1 – 18 years) were consecutively recruited in Austria to the international treatment trial AIEOP-BFM ALL 2000 (registered at http://www.clinicaltrials.gov/ as NCT00430118). Flow cytometric MRD assessment was done in these patients in the Vienna laboratory as part of a 4-center collaborative add-on study. Sampling for immunophenotypic and MRD investigations was approved along with the international trial by the institutional ethical committees, and was strictly done according to informed consent guidelines in accordance with the Declaration of Helsinki. In assessing CD20 expression we focused on the 237 cases with a CD10-positive BCP phenotype where we obtained enough material for analysis, thus excluding from further analysis 38 patients with T-ALL, 12 with a pro-B phenotype, and 19 with inadequate sample. Peripheral blood (PB) and bone marrow (BM) samples were investigated for this study at diagnosis and from several follow-up time-points during induction treatment: PB day 8, BM from days 15 and 33. Induction treatment consisted of a 7-day pre-phase with daily oral prednisolone (60 mg/m² body-surface-area/day) and one dose of intrathecal methotrexate (age-adjusted). From day 8 until day 33, patients received a four-drug regimen containing randomized glucocorticoids (prednisolone 60 mg/m² or dexamethasone 10 mg/m²), 8 infusions of L-asparaginase, four administrations of daunorubicin and vincristine, as well as additional intrathecals. Glucocorticoid medication was tapered after day 28. A second induction phase was started not earlier than on day 36.

Data on genotype, clinical risk stratification (low-, medium-, and high-risk) and outcome were retrieved from the Austrian ALL-BFM 2000 study office.
Flow cytometry

The standardized procedure has been recently described in detail including sample preparation, description of the entire antibody panel, stain-lyse procedure, and flow cytometric analysis. A fixed quadruple-stain was used to investigate CD20 expression: CD20/CD10/CD19/CD34, (ordered by channel 1 to 4: fluorescein isothiocyanate, FITC; phycoerythrin, PE; phycoerythrin-cyanin 7, PE-Cy7; allophycocyanin, APC). In experiments assessing differences in CD20 expression between viable and apoptotic blast cells, an Annexin V-staining reagent (Becton Dickinson; BD) was substituted for CD34 in channel 4. The instrument set-up was optimized daily by analyzing Calibrite™ beads (BD) and normal adult peripheral blood (PB) cells stained with CD4/CD8/CD3/CD45.

Immunophenotyping at diagnosis and quality control evaluations were performed collecting at least 30 000 cellular events, while for MRD measurements 300 000 events were acquired from 700 000 stained cells. Cell acquisition was performed with a FACSCalibur™ cytometer (BD) using the CELL Quest™ software (BD). Data analysis was done with the PAINT-A-GATE™ software (BD). Leukemic cells were identified using an immunological gate (associated with 90°-scattering, SSC) which included all CD19-positive cells. Minimal residual disease was defined as an accumulation of at least 10 clustered events displaying lymphoid scattering properties and leukemia-associated immunophenotypic characteristics as reported.

CD20 expression of samples was estimated by assessing the proportion of leukemic cells positive for the antigen with a cutoff of ≥20%. The threshold was set according to the upper limit of the background fluorescence of residual lymphoid cells not expressing
B-cell markers within the same acquisition. In addition, CD20 expression levels were quantified on the basis of mean fluorescence intensity (MFI) values using the CELL Quest™ software as reported.\textsuperscript{10,15} DAKO Fluorospheres™ (type IIIa beads) with assigned values of molecules of equivalent soluble fluorochrome were used for longitudinal monitoring of instrument performance stability.\textsuperscript{14} Measurements of these beads on an approximately weekly basis (which contain blank as well as fluorescence beads with five different emission intensities) were performed. These MFI-measurements gave standard deviations (SD; per five fluorescence levels of the beads) of ±12.4 to 14.9\% (min-max) in the FITC-channel per period of ≥12 months. These proportions reflect the low background of technical variance in MFI measurements during our study.

\textit{Rituximab experiments}

Paired BM samples from 9 patients from diagnosis and follow-up time-points during induction were selected for these experiments according to a negative/dim CD20-expression status at diagnosis, and to obvious CD20 up-regulation (various degrees) as well as sufficient MRD proportions at follow-up (≥1\%). Mono-nucleated cells were prepared by gradient separation (Lymphoprep®, Fresenius). Cells (approx. 1x10\(^6\) per test) were then incubated for one hour at 37\° C with human complement serum (15\% v/v; SIGMA) with or without clinical grade rituximab (Mabthera™; end-concentration 0.2 mg/mL), and with or without paired micro-antibodies (MB-55, anti-CD55, and MB-59, anti-CD59, end-concentration 13 µg/mL for both). Rituximab was kindly provided by P. Krenn and A. Wolfsberger (Vienna), and the micro-antibodies were a kind gift of P. Macor (Trieste, Italy).\textsuperscript{18} Notably, in tests investigating the effects of micro-antibodies
(“augmented lysis”), cells were pre-incubated with these for 30 minutes at 37°C to allow for antigen saturation before adding rituximab and complement. After incubations, cells were washed with cold PBS, transferred into Trucount™ tubes (BD) and stained for 15 minutes at r.t. with CD10/CD19/CD3/CD45 plus DAPI (for dead-cell exclusion). Without a further washing step, samples were acquired on a LSRII cytometer (BD; equipped with 3 lasers including one with 405nm emission). The remaining live leukemic blasts were assessed in absolute counts by recalculating the measured proportions using beads (of Trucount™ tubes) as internal standards. Rituximab-derived values were denoted as proportional recovery compared to control (i.e. incubations with complement alone).

**In-vitro incubations with glucocorticoids**

Mono-nucleated BM cells from 10 ALL patients at diagnosis were prepared by gradient separation as above and incubated with or without clinical grade prednisolone (0.00; 0.05; 0.5; 5 µg/mL) at 2x10⁵ cells per well (96-well plates) on human mesenchymal stroma cells using GIBCO™ AIM V medium without serum supplements (Invitrogen). As stroma cells we used a human BM mesenchymal cell line immortalized by enforced expression of telomerase which was kindly donated by D. Campana, St. Jude Children’s Cancer Research Hospital.¹⁹,²⁰ After three days of culture we assessed the CD20 expression (MFI values) on viable cells (mean recovery in controls: 66%; SD ±33%) in the different incubations using a CD20/CD10/CD19/Annexin V stain.

**Statistical analyses**

Concordance and correlations between sample cohorts or time-points were estimated using either the Shrout-Fleiss reliability assay or the Pearson correlation coefficient.
The Wilcoxon signed rank test (2-tailed) was used to assess the significance of observed differences between cohorts in paired data. Comparisons between the following data sets of paired BM or PB samples were performed: BM day0 vs. day15 (cBM0/15), PB day0 vs. day8 (cPB0/8), BM day0 vs. day33 (cBM0/33), and BM day15 vs. day33 (cBM15/33). The Chi-Square test was used to screen for differences in proportional distributions between cohorts. The Spearman rank correlation coefficient was used to assess the correlation between levels of CD20 expression (MFI values) and efficacy of rituximab-lysis.
Results

CD20 expression at diagnosis

We investigated 237 BM and 202 PB samples of CD10-positive BCP-ALL patients at diagnosis. We found expression of CD20 (≥20% positive blasts) in 46% (109 BMs) and 51% (104 PBs) of samples, respectively. The mean proportion of CD20\textsuperscript{pos} blasts per sample was low: 27% in BM (±29% SD; n=237) and 33% in PB (±32% SD; n=202). In paired analysis, there was a good correlation between the proportions of CD20\textsuperscript{pos} blasts in BM and PB (p<0.001; Pearson CC 0.83), but with some variance of values (mean difference 13% ±17% SD).

CD20 expression in follow-up

Paired samples from diagnosis and early follow-up, which had sufficient MRD (≥0.1%) to test reliably for CD20 expression, were available in 159 (BM from day 15; 67% of all) and 138 (PB from day 8; 68% of all) cases, respectively. Paired samples from diagnosis and late induction (BM day 33) had sufficient MRD in the latter in 27 cases (11%). Hence, all follow-up samples with very low or even negative MRD and their counterparts from diagnosis were excluded from further analyses. As compared to the total available sample cohort at diagnosis, those with paired follow-up samples had very similar proportions of CD20-positive cases at diagnosis (BM: 45%; PB: 52%). Follow-up specimens showed significantly increased CD20 expression when compared to their paired initial samples (Table 1). This up-modulation was seen as an overall increase in CD20-positive samples as early as at day 8 in PB (75% positive; cPB0/8: p<0.001), and also in follow-up BM samples (day 15: 71% positive, cBM0/15: p<0.001; day 33: 81%, cBM0/33 p<0.01). There was even a trend towards a further increase from
day 15 to day 33 (cBM15/33 p=0.05; n=25 pairs). The mean proportion of CD20<sup>pos</sup> blasts in BM was 25% at day 0 (±28% SD), rose to 47% at day 15 (±34%), and to 72% at day 33 (±36%). Regarding fluorescence levels, CD20 expression of blasts increased from a mean MFI-value of 14 in initial samples (±27 SD) to 45 (±100) at day 15, and to 109 (±187) at day 33. Only 5.7% of patients had a CD20-MFI > 50 at diagnosis, but the proportion increased to 22.2% at day 15 and to 48% at day 33.

In individual patients, the proportions of CD20<sup>pos</sup> blasts increased with time irrespective whether a given leukemic case at diagnosis had been CD20-positive or not (p<0.001 for all correlations; see Figure 1). The proportions of CD20<sup>pos</sup> blasts in paired PB and BM samples from days 8 and 15 correlated well (PCC 0.80; p<0.001), but with some variance (mean difference 15% ±18% SD). Notably, the CD20 expression neither correlated with amounts of MRD in day 8 PB (PCC 0.22) nor day 15 BM (PCC 0.18).

CD20 up-modulation occurs in viable cells

We recruited paired BM and PB samples from 10 consecutive patients with CD10-positive BCP-ALL to investigate and compare the levels of CD20 expression (MFI values) on viable (Annexin<sup>neg</sup>) and apoptotic (Annexin<sup>pos</sup>) leukemic blasts within the same specimens.

Apoptotic blasts showed slightly but significantly higher MFI values in the CD20 channel than viable cells, as reflected by a mean ratio of 1.28 (±0.3 SD; p<0.01) in BM samples at diagnosis and 1.32 (±0.34 SD; p=0.01) at day 15. Similarly, in PB samples we found mean increase factors of 1.32 (±0.76 SD; p<0.01) at diagnosis and of 1.17 (±0.21 SD; p=0.01) at day 8. However, compared to these differences between live and apoptotic cells the increases in CD20 expression on blast cells between diagnosis and follow-up
were much higher, and these latter differences were found when comparing viable as well as apoptotic cells separately. The mean increase factors in the BM samples (cBM0/15) were 5.84 (±7.0 SD; p<0.01) in viable blast cells and 5.32 (±6.0 SD; p<0.01) in apoptotic blast cells. Similarly, mean increase factors in PB samples (cPB0/8) were 4.46 (±5.2 SD; p<0.01) in Annexin\textsuperscript{neg} blasts and 4.40 (±4.7 SD; p<0.01) in Annexin\textsuperscript{pos} leukemia cells. All ratios built upon comparisons between cells at diagnosis and at follow-up were significantly higher than those from viable versus dead cell comparisons (p<0.05 and lower in Wilcoxon’s ranking test for paired data).

CD20 expression in sub-groups of patients

The two patient cohorts receiving different glucocorticoids (days 8 until 33) upon initial randomization were very similar in CD20 expression at baseline, and also reacted similar as estimated at day 15 and day 33 in BM (p=not significant).

There was no significant correlation between CD20 expression and age (as tested in groups of 1 – 5, 6 – 10, and >10 years) or ALL-phenotype (C- vs. preB-subtype). In addition, we found no significant difference in CD20 expression in BM samples between the three BFM risk groups (Table 1), neither at diagnosis (p=0.06) nor at day 15 (p=0.32). CD20 up-regulation occurred in follow-up in all three BFM risk groups (p<0.001 for each risk group), with high-risk patients showing very high proportions of positive cases at all time-points (83-88%).

TEL/AML1-rearranged cases (n=46) showed significantly lower CD20 expression in BM samples both at diagnosis and day 15 compared to the complementary cohort (n=113; p<0.01 for all correlations), as reflected by the number of CD20-positive cases (Table 1), proportions of CD20\textsuperscript{pos} blasts, and MFI values (data not shown). Hyperdiploid ALLs
(n=39) had higher CD20 expression than TEL/AML1-rearranged cases at day 15 (p<0.01 for all correlations), but at diagnosis only with respect to MFI values (p<0.01). Non-TEL/AML1//non-hyperdiploid samples (n=74) had higher CD20 values than TEL/AML1 cases in all items both at diagnosis and day 15 (p<0.01 in all correlations), but were not significantly different in similar comparisons from hyperdiploid cases. CD20 was up-regulated during induction both in TEL/AML1 and non-TEL/AML1 patients (p<0.01 for both; Table 1), but the former reached lower positivity (in 46-56% of cases) than the latter (81-85%).

Fourteen patients of the cohort of 159 had a relapse within a median observation period of 4.5 years. There was no significant correlation between the relapse status and CD20 expression of leukemic cells from BM, neither at diagnosis (p=0.17) nor at day 15 (p=0.32). Of the 14 relapse cases only four were CD20-positive initially as compared to 11 at day 15. The mean proportions of CD20pos blasts increased from 13% (±15% SD) at diagnosis to 44% (±28% SD) at day 15. The three relapsed patients, who were included in the group of 27 with high MRD also at day 33, had 75% (mean; ±17% SD) CD20pos blasts at this time-point.

CD20 up-regulation enhances rituximab-inducible complement-lysis

In 9 sample pairs of BM from diagnosis and follow-up we tested whether CD20 up-regulation is strong and specific enough to translate into relevant complement-dependent cytolysis induced by rituximab in in-vitro incubation. There were sufficient leukemic cells in these follow-up samples to do the test reliably (mean MRD-proportion 17.9% ±21.6% SD). The selected sample pairs showed low CD20 expression at diagnosis (mean MFI 20 ±12 SD) and significantly higher levels at follow-up (mean MFI
140 ±107 SD; p<0.01 for difference by Wilcoxon’s signed rank test). As exemplified in Figure 2, rituximab induced significantly more efficient cytolysis in follow-up samples (mean recovery 39% ±67% SD) than in initial specimens (mean recovery 82% ±23% SD; p<0.05 for difference). Stronger cytolysis was seen in all samples with CD20 MFI >50 as compared to those with lower MFI values (Figure 3A). Furthermore, we tested whether rituximab-action could be augmented by neutralizing the complement-regulatory proteins CD55 and CD59. Using blocking antibodies to these antigens in six sample pairs, the “augmented” lysis was slightly more efficient than rituximab-lysis alone in 11 of the 12 samples investigated (mean recovery 40% ±36% versus 51% ±38% SD; p=0.01; Figure 3B).

CD20 expression is modulated by prednisolone in-vitro

We incubated 10 initial ALL samples with various concentrations of prednisolone for three days and assessed thereafter the CD20 expression levels on viable (Annexin^neg) leukemic cells. Expression changes were compared with those found in the same patients in samples from diagnosis and day 8 of induction after only the prednisolone pre-phase (Figure 4). Four samples showed significant up-regulation in-vitro, and the three of these which had sufficient leukemic cells on day 8 for analysis also showed up-regulation in-vivo. Further six samples showed only minor or no modulation in-vitro, four of which (3 TEL/AML-1-rearranged cases) also showed no up-regulation in-vivo. Two TEL/AML-1-negative samples showed no up-regulation after three days in-vitro incubation, but clear up-modulation in-vivo at day 8.
Discussion

Immunophenotypic characterization of ALL is important for directing therapy and predicting outcome. Except for very recent developments, however, the antigenic peculiarities of ALL cells, as determinable by immunophenotyping, have not been exploited for direct therapeutic interventions; this situation is about to change.

CD20, an important differentiation-related surface antigen of B-cells, is commonly employed as a marker in leukemia phenotyping as well as for assessment of MRD in BCP-ALL. Its expression has been associated with a better, or an inferior outcome in ALL, mostly likely depending on the treatment used. With substantial similarity between observations, CD20 expression (based on either a cutoff of 20% positivity or relevant fluorescence intensity) was found in about 48% of patients with BCP-ALL, which favorably compares to the proportion noted in our present study (46% of initial BM samples). There was no significant relation of CD20 expression to outcome in terms of relapses in our BFM-protocol-based patient cohort with BCP-ALL. In addition to these results, we observed in many patients and among all risk groups that CD20 expression is significantly up-regulated on leukemic cells during the phase of induction chemotherapy. Since CD20 is the target of well established immunotherapeutic agents like rituximab, our observation appears relevant towards using antigenic patterns of leukemia for treatment. Two aspects seem important herein. The first is that leukemic cells can be influenced to alter their cellular processes and to acquire qualities exploitable for their eradication. There is now sufficient evidence to consider that leukemic phenotypes (of individual clonal cases) are not stable but may vary within certain limits upon outer influences. In this respect, these changes in
leukemia phenotypes may well be considered just as surrogate appearance of the cellular processes in general.\textsuperscript{12} This property could be deployed for future treatment specifically in cases where certain sub-cellular pathways, as e.g. those leading to apoptosis, are blocked or insufficient, so that leukemia could be tackled via alternative routes. Importantly, we found that in particular high-risk BCP-ALLs (which include those with a poor glucocorticoid response or even with a poor response to a four-drug induction) were CD20-positive in follow-up in 83\% of cases. More generally, 81\% of all patients with significant MRD after induction (\(\geq 0.1\%\)), an amount known to correlate with dismal outcome,\textsuperscript{16} were CD20-positive even at quite high levels of intensity (day 33: mean MFI 109 \(\pm 187\) SD). In the in-vitro experiments we found efficient cytolysis only when CD20 expression intensities surpassed a MFI value of 50. This corresponds in our system roughly to \(\geq 80\text{-}90\%\) positive blasts: i.e. a fluorescence distribution of leukemic cells almost non-overlapping with control cells. Hence, mere CD20-positivity at diagnosis based on the cut-off of \(\geq 20\%\) of blasts positive with the marker was found insufficient to predict cytolysis in-vitro. Nevertheless, the up-regulation seen in follow-up was specific and efficient enough to translate into relevant rituximab-activity. Hence, using a cutoff of \(\geq 90\%\) or \(\geq 80\%\) positive cells, 52\text{-}67\% of the MRD-positive (\(\geq 0.1\%\)) patients with BCP-ALL in our series met the criteria at end-induction, as opposed to only about 5\text{-}8\% of cases at diagnosis and 20\text{-}25\% at day 15, respectively. Notably, a dependency of rituximab-inducible complement-lysis on levels of CD20 expression was reported also in B-cell chronic lymphocytic leukemia.\textsuperscript{25} Compared to the latter study, we used a higher rituximab concentration similar to that reached in serum after a single standard in vivo application of 375 mg/m\(^2\).\textsuperscript{26} However, complement-lysis is not the only mechanism by which rituximab induces cytotoxicity.\textsuperscript{27} It may therefore not be possible to deduce an
algorithm from our in-vitro results which would allow determining the potential in-vivo success of rituximab in ALL.

The second important aspect is that this intervention, which influences the leukemic cellular processes in a way that blast cells acquire qualities exploitable for their eradication, comes along without a need for any extra medication. The process seems to be induced by the induction chemotherapy for ALL, and since obvious as early as at day 8 of therapy (after one week of prednisolone and a single intrathecal methotrexate instillation) most probably is triggered in this phase by glucocorticoids.\textsuperscript{12} This is corroborated by our finding that CD20 is up-modulated by prednisolone incubation in-vitro, an effect which we found to be non-uniform and patient-specific. We are aware that after the prednisolone-pre-phase, also other medications may add to the observed effect. Other substances than steroids have already been shown to influence CD20 expression. For example, bryostatin-1, a protein kinase C modulator, enhances both CD20 mRNA and protein levels, and a combined therapy with rituximab was suggested recently.\textsuperscript{28} In cell line experiments this effect was found to be even insensitive to steroid action. Such effects add to strategies aiming at enhancing anti-CD20 directed cytotoxicity. The Memorial Sloan-Kettering Cancer Center currently screens the possibility to use orally available beta-glucans, which prime complement binding to the cell surface and thus enhance rituximab activity.\textsuperscript{29} Increasing complement-lysis by neutralizing the inhibitory surface molecules CD55 and CD59,\textsuperscript{18,25} or by driving CD20 to segregate into lipid rafts via cross-linking by a new polymer formulation of rituximab are further evaluated strategies.\textsuperscript{30,31} Notably, other anti-CD20 antibodies, like tositumomab, may have higher affinity to CD20 as well as different mechanisms of action compared to rituximab.\textsuperscript{32} Immunotherapy directed to other antigens, like CD52, also complements the
novel armamentarium against lymphoid malignancies. Target validation on a case-by-case basis has been suggested in this context. Of note, the TEL/AML1-rearranged cases in our study had much lower CD20 expression at baseline and also poorly up-regulated the antigen in follow-up. Of 46 TEL/AML1 cases, none at diagnosis, only one at day 15 (2%), and also not the single case with relevant MRD at day 33 showed CD20 expression on $\geq 90\%$ of blasts, as opposed 7%, 27%, and 54% of the non-TEL/AML1 cases at these time-points. These results together with the observations of Golay et al., who showed that TEL/AML1 cases are very sensitive to anti-CD52 treatment as opposed to non-TEL/AML1 leukemias, create the possibility to choose rituximab or alemtuzumab based on genotype.

In summary, we have shown that CD20 is up-regulated during induction therapy with BFM-type protocols in a very significant proportion of patients with non-TEL/AML1 BCP-ALL. Most relevantly, the process is continued and sustained until end-induction (and even thereafter; M.N.D., manuscript submitted August 2008) so that particularly patients with high MRD at this time-point could profit from anti-CD20 directed immunotherapy. Since up-regulation of CD20 is induced during chemotherapy (most obviously due to glucocorticoid action) without a need for extra medication, this is a simple and straightforward way to influence leukemic cells so that they acquire qualities exploitable for their eradication. Based on this rationale, clinical trials in pediatric ALL could now be designed to assess whether rituximab would result in a clinical benefit.
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Authorship

Dworzak: Designed research, collected data, analyzed and interpreted data, drafted the manuscript

Schumich: Did most sample analyses and experimentation

Printz: Provided experimental guidance

Pötschger: Performed statistical analyses

Husak: Contributed in analyses and experimentation

Attarbaschi: Contributed in analyses and experimentation

Basso: Designed research, participated in manuscript drafting

Gaipa: Designed research, participated in manuscript drafting

Ratei: Designed research, participated in manuscript drafting

Mann: Collected samples and provided clinical data

Gadner: Designed research and secured funding

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References


Figure legends

Figure 1. **Proportions of CD20-positive blasts in paired samples from diagnosis and follow-up.** Comparisons of PB samples from day 0 versus day 8 (left plot), as well as of BM samples from diagnosis versus day 15 (center) and day 33 (right) are shown. Each data point represents one BCP-ALL case. Thresholds for determining CD20 expression on leukemic cells were set by using the background fluorescence of residual non-B lymphocytes within the same acquisition. Follow-up samples only with ≥0.1% residual leukemic cells were analyzed. Dashed lines mark the cutoff of 20% used to describe a sample as positive or negative. The numbers of samples located within each of the four quadrants, which are built by the cutoff lines, are shown. Identical results in paired samples would fit closely to the dotted diagonal line. In all comparative plots it can be seen that follow-up samples have higher proportions of CD20<sup>pos</sup> leukemic cells than the paired samples at diagnosis.

Figure 2. **CD20 up-regulation in follow-up translates into efficient rituximab-induced complement-lysis.** Dot plots are derived from analyses of paired BM samples of a high-risk BCP-ALL patient at diagnosis and at end-induction. Phenotypic comparisons and cell recovery analyses after in-vitro incubations with complement alone, with complement plus rituximab (0.2 mg/mL), and with complement plus rituximab plus micro-antibodies neutralizing CD55 and CD59 are shown. The dashed arrow points at the cluster of Trucount<sup>™</sup> beads which were used as internal standards for absolute cell recovery assessment. Up-regulation of CD20 expression can be seen on the CD10<sup>pos</sup> leukemic cells (black) which remained after therapy. An almost complete
reduction of intact (DAPI-negative) CD10<sup>pos</sup> leukemic cells can be seen upon rituximab incubations, with a small remnant fraction of viable leukemic cells marked with an arrow. Of note, cells lysed by complement mostly disappear from dot plots upon severe cellular disruption.

**Figure 3.** Efficacy of rituximab-induced complement-lysis increases with higher intensity of CD20 expression (A) and with inhibition of complement-regulatory antigens (B). In (A) 18 samples (9 pairs) of BCP-ALL at diagnosis (open dots) and from follow-up (black dots) were analyzed for CD20 expression levels (MFI values) and blast cell recovery upon in vitro rituximab/complement incubations. The (continuous) regression line and the MFI channel = 50 (dotted line) are shown. Note that all but one follow-up sample show higher CD20 expression than initial samples along with more efficacious rituximab-lysis (best separator apparently at MFI 50). In (B) comparisons of complement-lysis efficacy with rituximab alone versus rituximab plus additional mini-antibodies against CD55 and CD59, denoted “augmented” lysis, are shown. Twelve samples (from six of the 9 pairs, as above) were tested. Differences between incubations were minor but statistically significant.

**Figure 4.** Prednisolone increases CD20 expression in-vitro. CD20 expression levels (MFI values) of viable leukemic cells of 10 ALL samples after incubation for three days with various concentrations of prednisolone (none; 0.05 to 5 µg/mL) are shown (individual patients are characterized by specific symbols). In-vivo expression changes in samples taken at diagnosis and day 8 (after the prednisolone pre-phase) among the same patients are shown for comparison. Note patient-specific modulation patterns.
Figure 1.

Figure 2.
Figure 3.

Figure 4.
Table 1. Proportions and absolute number of CD20-positive ALL cases

<table>
<thead>
<tr>
<th>CD20pos</th>
<th>N=</th>
<th>all with MRD</th>
<th>Low Risk</th>
<th>Medium Risk</th>
<th>High Risk</th>
<th>TEL/AML1</th>
<th>non-TEL/AML1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM day 0</td>
<td>159</td>
<td>45% 71 / 159 *(52%; 14 / 27)</td>
<td>51% 18 / 35</td>
<td>38% 38 / 100</td>
<td>63% 15 / 24</td>
<td>28% 13 / 46</td>
<td>51% 58 / 113</td>
</tr>
<tr>
<td>PB day 0</td>
<td>138</td>
<td>52% 72 / 138 *(58%; 14 / 23)</td>
<td>55% 18 / 33</td>
<td>48% 42 / 88</td>
<td>71% 12 / 17</td>
<td>24% 8 / 34</td>
<td>62% 64 / 104</td>
</tr>
<tr>
<td>PB day 8</td>
<td>138</td>
<td>75% 104 / 138 *(79%; 19 / 24)</td>
<td>76% 25 / 33</td>
<td>73% 64 / 88</td>
<td>88% 15 / 17</td>
<td>56% 19 / 34</td>
<td>82% 85 / 104</td>
</tr>
<tr>
<td>BM day 15</td>
<td>159</td>
<td>71% 113 / 159 *(76%; 19 / 25)</td>
<td>66% 23 / 35</td>
<td>70% 70 / 100</td>
<td>83% 20 / 24</td>
<td>46% 21 / 46</td>
<td>81% 92 / 113</td>
</tr>
<tr>
<td>BM day 33</td>
<td>27</td>
<td>81% 22 / 27</td>
<td>100% 1 / 1</td>
<td>79% 11 / 14</td>
<td>83% 10 / 12</td>
<td>0% 0 / 1</td>
<td>85% 22 / 26</td>
</tr>
</tbody>
</table>

* results from patients which could be analyzed also at d33
CD20 up-regulation in pediatric B-cell precursor acute lymphoblastic leukemia during induction treatment: setting the stage for anti-CD20 directed immuno-therapy

Michael N. Dworzak, Angela Schumich, Dieter Printz, Ulrike Potschger, Zvenyslava Husak, Andishe Attarbaschi, Giuseppe Basso, Giuseppe Gaipa, Richard Ratei, Georg Mann and Helmut Gadner