Neutralization of membrane complement regulators improves complement-dependent effector functions of therapeutic anticancer antibodies targeting leukemic cells

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ALM, Alemtuzumab; CDC, complement-dependent cytotoxicity; CDCC, complement-dependent cellular cytotoxicity; MAC, membrane attack complex; mCRP, membrane-bound complement regulatory protein; NHS, Normal Human Serum; OFA, Ofatumumab; PBLs, peripheral blood leukocytes; RTX, Rituximab; siRNA, small interfering RNA; TRX, Trastuzumab

Complement-dependent cytotoxicity (CDC) is one of the effector mechanisms mediated by therapeutic anticancer monoclonal antibodies (mAbs). However, the efficacy of antibodies is limited by the resistance of malignant cells to complement attack, primarily due to the over-expression of one or more membrane complement regulatory proteins (mCRPs) CD46, CD55, and CD59. CD20-positive Burkitt lymphoma Raji cells and primary CLL cells are resistant to rituximab (RTX)-induced CDC whereas ofatumumab (OFA) proved to be more efficient in cell killing. Primary CLL cells but not CD52-positive acute lymphoblastic leukemia (ALL) REH cells were sensitive to alemtuzumab (ALM)-induced CDC. Upon combined inhibition on Raji and CLL cells by mCRPs-specific siRNAs or neutralizing antibodies, CDC induced by RTX and by OFA was augmented. Similarly, CDC of REH cells was enhanced after mCRPs were inhibited upon treatment with ALM. All mAbs induced C3 opsonization, which was significantly augmented upon blocking mCRPs. C3 opsonization led to enhanced cell-mediated cytotoxicity of leukemia cells exposed to PBLs or macrophages. Furthermore, opsonized CLL cells were efficiently phagocytized by macrophages. Our results provide conclusive evidence that inhibition of mCRPs expression sensitizes leukemic cells to complement attack thereby enhancing the therapeutic effect of mAbs targeting leukemic cells.

Introduction

As an essential component of the host defense, complement is involved in the elimination of infectious pathogens and in the removal of immune complexes and apoptotic cells.1,2 Complex activation leads to the generation of biologically active macromolecules and peptides that exert multiple effector functions such as cytotoxicity, chemotaxis, opsonization, and phagocytosis.3-5 Antibody-mediated activation of complement on a tumor cell results in three main effector mechanisms: (a) opsonization of target cells with C3 fragments,6 (b) recruitment and activation of various immune cells by anaphylatoxins C3a and C5a,7 and (c) finally the formation of the membrane attack complex (MAC) that leads to tumor cell lysis, referred to as CDC.8 Apart from direct CDC, cell bound C3 metabolites (iC3b/C3d) are recognized by complement receptor 3 (CR3, CD11b/CD18) on immune effector cells enabling complement-dependent cellular cytotoxicity (CDCC) and augmenting antibody-dependent cellular cytotoxicity (ADCC).8,9 Cumulative data indicate that a constitutive elevated complement resistance of tumor cells to CDC hampers the efficacy of therapeutic antibodies in cancer therapy.10,11 This is primarily due to the overexpression of the membrane-bound complement regulatory proteins (mCRPs) decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), and CD59 on malignant cells, which normally protect all host cells from accidental complement attack.10,12,13 Inhibition of mCRPs by neutralizing antibodies or posttranscriptional gene silencing increases CDC of various tumor cells.14-17

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Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of long-lived clonal B-lymphocytes. Despite much progress in therapeutic treatments, the disease is still incurable. mAbs against CD20 and CD52 revealed good therapeutic potential for CLL. Rituximab (RTX), a chimeric monoclonal anti-CD20 antibody, was the first antibody to be approved for the treatment of lymphomas and is also clinically used for several B-cell neoplasias.\textsuperscript{18-20} The main antitumor effector mechanisms mediated by RTX in killing lymphoma cells include ADCC and CDC.\textsuperscript{21} Despite its therapeutic success, RTX has shown limited efficacy as a single agent, especially in CLL and mantle cell lymphoma.\textsuperscript{22} It appears that complement resistance protects various B-cell lymphoma from RTX-mediated killing.\textsuperscript{21-23} Overexpression of CD59 has been shown to limit rituximab therapy in patients with various B-cell malignancies.\textsuperscript{24} Inhibition of CD55 and CD59 expression sensitized rituximab-resistant lymphoma cells to CDC.\textsuperscript{25,26} To further improve the therapeutic options for CD20-positive B-cell malignancies, several new or modified anti-CD20 antibodies have been developed.\textsuperscript{27} Ofatumumab (OFA; Arzerra) is a fully human IgG1\textsubscript{k} monoclonal antibody approved for treatment of patients with CLL. OFA binds to a different epitope on CD20 and is more efficient in activating complement as compared to RTX.\textsuperscript{28,29} Besides membrane bound complement regulators, factor H, a fluid-phase complement regulator also contributes to the resistance of CLL cells to complement attack and inhibition of factor H binding to CLL cells improves rituximab and OFA-mediated CDC.\textsuperscript{30,31}

ALM, a humanized IgG1\textsubscript{k} anti-CD52 antibody, has shown therapeutic activity in CLL and has been approved for the treatment of fludarabine-resistant patients.\textsuperscript{32-34} The mechanism of action of ALM is mainly through ADCC\textsuperscript{35} and CDC.\textsuperscript{36} ALM has been shown to lyse ALL cells and further blocking of CD55 and CD59 expression improved its biological activity.\textsuperscript{37}

Thus, enhancement of immune-mediated effector functions will certainly improve the efficacy of therapeutic antibodies.\textsuperscript{8,38} We have recently demonstrated that silencing of membrane regulators significantly enhances complement-dependent antitumor activity of trastuzumab (TRX) and pertuzumab.\textsuperscript{39} Only few reports have demonstrated that neutralization of complement regulators either by blocking antibodies or by inhibitors sensitize leukemia cells to RTX-mediated CDC.\textsuperscript{25,26} However, to our knowledge, no study has reported the impact of silencing of complement regulators on complement-dependent cell mediated cytotoxicity (CDC) induced by RTX or OFA or ALM on leukemia cells. In the present study, we provide evidence for the first time that neutralization of mCRPs expression on leukemia cells either by siRNA or by blocking antibodies not only enhances CDC and opsonization but also increases CDC by PBLs and macrophages.

### Results

**siRNA-induced silencing of mCRP expression**

Raji cells derived from Burkitt lymphoma and REH cells derived from ALL were chosen as representative lymphoma cell lines for our study. In addition, primary human CLL cells from 11 patients were used (Table 1). Flow cytometry analysis showed varying expression of CD20 and CD52 as well as of the complement regulators CD46, CD55, and CD59 on Raji cells, REH, and CLL cells (Fig. 1).

Due to the fact that primary B-lymphocytes and B-cell lines are extremely resistant to most lipid based gene transfer techniques,\textsuperscript{40} we used a nucleofection-based strategy to transfect Raji and REH cells with siRNAs against CD46, CD55, and CD59 alone or in combination. CD46 expression was reduced by 44 ± 14% in Raji and by 45 ± 10% in REH cells. The expression of CD55 was inhibited by 58 ± 7% in Raji and by 53 ± 14% in REH cells. CD59 expression was downregulated by 54 ± 5% in Raji and by 69 ± 9% in REH cells. The combined transfection of the siRNAs yielded a slightly reduced, but significant reduction of each individual target protein (Fig. 2). Due to high toxicity, primary CLL cells were not transfected with siRNA using nucleofection (data not shown).

**Neutralization of complement regulators augments CDC**

OFA was superior compared to RTX in lysing Raji cells. Lysis of non-transfected Raji cells was 41 ± 4% with RTX and 71 ± 3% with OFA. Selective inhibition of CD55 or CD59 on Raji cells further increased cell lysis by 16 ± 3% and 17 ± 2%, respectively, upon incubation with RTX, but not with OFA. Inhibition of CD46 alone had no effect, whereas the combined downregulation of all three regulators further enhanced overall cell lysis by 47 ± 11% and 22 ± 8% upon treatment with RTX or OFA, respectively (Fig. 3A). Addition of heat inactivated NHS instead of NHS completely abolished cell lysis (data not shown). In contrast to Raji cells, CLL cells were highly resistant to RTX-induced CDC (10 ± 8%) whereas OFA was more effective (63 ± 12%). Due to the high toxicity of siRNA with nucleofection on CLL cells, non-complement fixing neutralizing antibodies against CD46, CD55, and CD59 were employed. Blocking individual complement regulators variably increased RTX-induced CDC (data not shown). The combined inhibition of all three regulators further augmented CDC by 63% or 23% induced by RTX or OFA, respectively. Incubation with the control anti-HER2 antibody TRX had no effect (Fig. 3C).

### Table 1. Characteristics of CLL patients used in the study

| Patient | Age | Cytogenetics | Mutations | IGHV |
|---------|-----|--------------|-----------|------|
| CLL-1   | 70  | 11q-,-13q-   | SF3B1, TP53 | M    |
| CLL-2   | 77  | 17p-         | SF3B1, TP53 | U    |
| CLL-3   | 68  | 13q-,17p-    | BRF, SF3B1, TP53 | U |
| CLL-4   | 73  | 13q-         | none       | M    |
| CLL-5   | 63  | 13q-         | none       | M    |
| CLL-6   | 58  | 13q-         | none       | U    |
| CLL-7   | 59  | 13q-         | none       | M    |
| CLL-8   | 87  | 17p-         | TP53       | U    |
| CLL-9   | 50  | 13q-         | none       | M    |
| CLL-10  | 81  | 17p-         | TP53       | M    |
| CLL-11  | 55  | 13q-         | none       | M    |
In the ALL cell line REH, we observed 31 ± 4% CDC by ALM. Downregulation of CD59 expression resulted in 10 ± 7% increased cell lysis, whereas inhibition of CD46 or CD55 alone had no significant effect. Most pronounced was the effect of a combined inhibition of CD46, CD55, and CD59 expression resulting in an overall cell lysis of 51 ± 11% in REH cells (Fig. 3B). As ALM was already highly effective in lysing CLL cells leading to 76 ± 18% cell lysis, blocking of all the three regulators only slightly increased cell lysis by 13% (Fig. 3C).

Enhanced opsonization of leukemic cells

We then analyzed the efficacy of RTX or OFA to induce C3 opsonization on Raji cells. We observed a higher iC3b deposition with OFA than with RTX (Fig. 4A). In the presence of RTX, knockdown of CD55 alone increased iC3b deposition by 36% (MFI 379 ± 30 vs. MFI 516 ± 32). Inhibition of CD46 or CD59 had no significant effect. Inhibition of individual complement regulators had also no effect on iC3b deposition induced by OFA. In contrast, increased iC3b deposition by 53% (MFI 379 ± 130 vs. MFI 581 ± 40) was observed after combined downregulation of all three regulators in the presence of RTX and by 71% (MFI 541 ± 20 vs. MFI 923 ± 159) with OFA (Fig. 4A). Similarly, OFA activated complement more efficiently than RTX on CLL cells as reflected by significant higher C3d deposition. Combined blocking of CD46 and CD55 significantly augmented C3d deposition by 137% (MFI 251 ± 117 vs. MFI 595 ± 392) in the presence of RTX and by 83% (MFI 919 ± 565 vs. MFI 1678 ± 712) if induced by OFA (Fig. 4C).

On REH cells, ALM induced iC3b deposition (MFI 106 ± 26) was increased upon silencing of CD46 and CD55 by 52% and 53%, respectively. The effect was even more pronounced upon the combined knockdown of all three regulators with an increase in iC3b deposition by 86% (Fig. 4B). ALM activated complement on CLL cells with a higher efficiency (MFI 1450 ± 1092) than on REH cells. Blocking of CD46 and CD55 further increased C3d deposition by 38% (MFI 2000 ± 1274) (Fig. 4C).

Improved ADCC of leukemic cells

RTX and OFA are known to induce ADCC, but a possible additional effect of complement or mCRP neutralization on NK-cell mediated lysis has not yet been investigated. To address this question, Raji cells were either transfected with a combination of all the three mCRP-specific siRNA or with control non-targeted siRNA or left untreated before incubation with RTX or OFA. To allow C3 deposition and to avoid MAC formation, C8 depleted human serum was added and opsonized cells were incubated with PBLs. RTX and OFA induced ADCC with similar efficiency (Fig. 5A and B). Due to a better C3 opsonization, OFA induced a higher CDCC (71 ± 7%) than RTX (43 ± 5%). Knockdown of mCRP's expression further augmented cell lysis by 81 ± 8% with RTX and 99 ± 12% with OFA. Similar observations were made on REH cells with ALM, where cell lysis was further increased to 33 ± 10% upon silencing of mCRP's expression (Fig. 5C). Pre-incubation with inactivated serum completely abolished PBL mediated cell lysis (Fig. 5A–C).

RTX and OFA were similarly efficient in inducing ADCC of CLL cells (Fig. 5D). Blocking of CD46 and CD55 significantly augmented RTX-induced PBL mediated lysis up to 60%. If C3 opsonization was induced by OFA, PBL yielded significant cell lysis, which, as expected, was further augmented up to almost 90% after blocking of CD46 and CD55. The best C3 opsonization effect was achieved by ALM leading to a CDCC of 83 ± 15%. Upon inhibition of CD46 and CD55, 100% cell lysis was achieved (Fig. 5D). This clearly indicates that complement improves PBL-mediated ADCC and that mCRPs neutralization further augments this effect.

Complement-dependent macrophage mediated cytotoxicity of leukemic cells

C3 opsonized Raji and REH cells were also exposed to macrophages that were differentiated in the presence of IFNγ or M-CSF. Both sub-populations of macrophages were able to lyse the...
tumor cells, however with varying efficacy (Fig. 6A and 6B). Knockdown of all three regulators led to an increased cell-mediated cytotoxicity of Raji cells by 14% and 13% in the presence of IFN\(\gamma\) or M-CSF differentiated macrophages, respectively (Fig. 6A). Similarly, we obtained an augmented lysis of REH cells by 26% and 24% in the presence of IFN\(\gamma\) or M-CSF differentiated macrophages, respectively, upon mCRPs knockdown (Fig. 6B). Cell lysis was negligible in the absence of macrophages or in the presence of inactivated serum.

Primary CLL cells were equally sensitive to macrophage-mediated antibody dependent cellular cytotoxicity (Fig. 6C). However, compared to PBL-mediated ADCC, pre-incubation of macrophages with complement and RTX did not yield significant cell lysis. Blocking of CD46 and CD55 significantly increased RTX-induced macrophage mediated lysis up to 50%. C3 opsonization induced by OFA yielded a significant macrophage mediated cell lysis up to 50% and, as expected, blocking CD46 and CD55 further augmented lysis up to 90%. ALM was more efficient in inducing Ab-dependent macrophage-mediated cell lysis (28±9%). C3 opsonization augmented macrophage-mediated cell lysis up to 83±26%, which increased up to 100% after blocking CD46 and CD55 (Fig. 6C). Thus, C3 opsonization contributes to the antitumor activity of macrophages and can be further improved by mCRP neutralization.

**Phagocytosis of opsonized CLL cells**

To investigate phagocytosis of C3 opsonized tumor cells, M-CSF differentiated macrophages were labeled with CFSE (green)
and CLL cells were stained with PKH26 (red) and analyzed by fluorescence microscopy. CLL cells treated with OFA and complement were more efficiently engulfed by macrophages than RTX and complement treated cells (36 ± 9% vs. 28 ± 9% respectively) (Fig. 6D and E). Blocking CD46 and CD55 significantly enhanced phagocytosis of RTX and OFA treated cells by 40 ± 13% and 55 ± 9% respectively (Fig. 6D and E). More efficient phagocytosis was achieved when CLL cells were treated with ALM (47 ± 13%). Here, neutralization of CD46 and CD55 increased phagocytosis to 70 ± 9%. (Fig. 6D and E). Blocking CD46 and CD55 antibody alone or pre-incubation of cells with inactivated serum did not influence phagocytosis (Fig. 6E).

Discussion

In recent years mAbs have been established as “standard of care” agents for several human cancers.41,42 Although complement is one of the effector systems involved in the antitumor activity of mAbs,8,43 overexpression of complement regulatory proteins by tumor cells restrict their therapeutic potential.10,12,44 We have previously shown that silencing membrane complement regulators enhances the therapeutic effect of TRX and pertuzumab on Her2/neu positive tumor cells.39 Here, we demonstrate that neutralizing mCRPs not only enhances CDC but also improves CDCC of malignant B-cells by PBLs and macrophages induced by two anti-CD20 antibodies, RTX and OFA, and by the anti-CD52 antibody, ALM.

Raji and CLL cells are more efficiently lysed by OFA than by RTX, which is consistent with previous reports.27,29 OFA binds to the small extracellular loop of CD20 in close proximity to the cell membrane with a slower off-rate than RTX28; C1q is bound with higher avidity and in close proximity to OFA, resulting in effective complement activation.45 Various studies have reported that complement regulatory proteins significantly limit CDC

![Figure 3](https://www.tandfonline.com/e979688-5)
of lymphoma cells upon RTX\textsuperscript{21,25,30} as well as OFA\textsuperscript{29,31,46} treatments. Similarly, ALM is effective in inducing CDC of CLL cells as well as of ALL cells and blocking CD55 and CD59 partly increased lysis.\textsuperscript{37} CDC mediated by ALM on CLL cells was further enhanced by the treatment with OFA, which may improve the clinical response to CLL cells resistant to CDC\textsuperscript{47}.

In this study, we applied RNA interference (RNAi)\textsuperscript{48} to knockdown the expression of CD46, CD55, and CD59 on B-cell lines. A great number of lipid based-strategies have emerged recently for efficient delivery of siRNA \textit{in vitro} and \textit{in vivo}. However, in line with published data,\textsuperscript{40} our various attempts for gene knockdown on B-cell lines and primary CLL cells by standard lipid based strategies failed (data not shown). To circumvent the lipid based transfection resistance, we used nucleofection, an advanced form of electroporation, to introduce siRNA into B-cell lines. Nucleofector technology is a highly efficient non-viral gene transfer method for hard-to-transfect cell lines.\textsuperscript{49} We achieved 50–60\% silencing of mCRPs expression in Raji and REH cell lines (Fig. 2). However, considerable toxicity of nucleofection to primary CLL excluded its application. Silencing of individual complement regulators exerted only a minor effect on CDC. To some extent, inhibition of CD59 alone was sufficient to sensitize Raji, REH, and CLL cells to RTX or ALM induced CDC respectively, but had no effect on OFA treatment. The most prominent effect was observed upon neutralization of all three regulators, where we observed increased lysis upon treatment with any of the mAbs.

The biological activity of complement is not restricted to CDC but also equally reflected by opsonization of target cells, improving cell-mediated defense mechanisms. Similar to CDC, OFA had a greater opsonizing capacity than RTX on both Raji and CLL cells and neutralizing CD46 and CD55 further improved C3 deposition. C3-derived opsonins are recognized by complement receptors (CR3, CD11b/CD18) on immune effector cells such as
macrophages, NK-cells, and PMNs to mediate and enhance CDCC and ADCC.\(^6,8\) In a murine model of metastatic cancer Elvington et al. showed that a CR2Fc construct significantly enhanced the outcome of mAb therapy via both macrophage-dependent FcγR-mediated ADCC and by direct CDC.\(^9\) In contrast, complement was shown to inhibit NK cell activation by RTX-coated target cells suggesting that C3b on target cells may disturb the interaction between the Fc part of RTX and NK-cell CD16.\(^50\) We here demonstrate that C3 opsonization further enhances ADCC of opsonized B-cells. In contrast to RTX, in the presence of OFA as well as of ALM ADCC of CLL cells were improved (Fig. 5D, bar 7 vs. bar 12 and 17), reflecting a stronger opsonizing capacity of those antibodies. Neutralization of mCRPs enhanced ADCC in presence of serum, correlating with increased C3 deposition.

As macrophages have been implicated as major players in RTX mode of action,\(^51,52\) we then investigated the effect of complement on macrophage-mediated cytotoxicity. Consistent with our previous observation on HER2-positive tumor cells,\(^39\) we found an increased killing of B-cells in the presence of complement (Fig. 6A and B). In the absence of complement, a comparable macrophage-mediated CLL cytotoxicity of OFA and RTX was observed (Fig. 6C, bar 5 and 9). However, C3 opsonization by OFA as well as ALM was superior to that of RTX as reflected by higher CDCC mediated by those antibodies (Fig. 6C, bar 7 vs. bar 11 and 16). In addition, enhanced CDCC of Raji and REH upon mCRP's silencing and reduced lysis in the presence of inactivated serum further support our observation. Finally, phagocytosis of CLL cells by macrophages was clearly improved upon C3 opsonization and could be further enhanced when mCRPs were neutralized (Fig. 6 D and E).

With these experiments, we provide further evidence that knockdown of complement regulators not only improves CDC and opsonization...
but also enhances ADCC and CDCC mediated by PBLs and macrophages. This leads to an improved therapeutic potential of anticancer antibodies targeting leukemic cells. The obstacle of this approach for clinical application is still the targeted delivery of specific siRNA to the tissue of interest. The feasibility of such a strategy has recently been demonstrated by our group showing an improved CDC upon specific delivery of siRNA using transferrin-coated lipoplexes. A successful targeted delivery of mCRPs-specific siRNAs may serve as an adjuvant to improve the efficacy of antibody-based immunotherapy.

**Materials and Methods**

**Cell lines and primary CLL cells**

The human Burkitt lymphoma cell line, Raji (DSMZ (German Collection of Microorganisms and Cell Cultures) Number: ACC 319) and the human ALL cell line REH (DSMZ, kindly provided by PD. Dr. Reinhard Schwartz-Albiez, Translational Immunology, DKFZ, Heidelberg, Germany) were cultured in RPMI 1640 with L-glutamine (PAA, Colbe, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS, Invitrogen, Karlsruhe, Germany) and maintained at 37°C, 5% CO2. All the cell lines used in the study were authenticated using Multiplex Cell Authentication by Multiplexion (Heidelberg, Germany). To obtain primary CLL cells, blood samples of CLL patients were collected after informed consent and the mononuclear cell fraction was purified by standard Ficoll Hypaque gradient centrifugation. The study was approved by the ethics committee of the University Hospital Heidelberg.

**Antibodies and other reagents**

Rituximab (RTX; Mabthera, anti-CD20 antibody) and Trastuzumab (TRX; Herceptin, anti-HER2/neu) were obtained from Roche Diagnostics GmbH (Penzberg, Germany), Ofatumumab (OFA; Arzerra, anti-CD20) was from GlaxoSmithKline (Brentford, UK) and Alemtuzumab (ALM; Campath-1H, anti-
CD52) from Berlex Laboratories (Montville, NJ, USA).

Monoclonal mouse anti-human CD46 antibody (IgG1, clone GB24) was kindly provided by Dr. J. Atkinson (Washington University, St. Louis, MO, USA). Mouse anti-human CD55 (IgG1, clone Bric 110), and mouse anti-human CD59 antibodies (IgG2b, clone Bric 229) were from International Blood Group Reference Laboratory (Birmingham, UK). For the detection of surface bound complement C3, monoclonal mouse anti-human iC3b (IgG1, clone A207, Quidel, San Diego, CA, USA), or a rabbit polyclonal anti-human C3d antibody (Dako, Hamburg, Germany) was used. Goat anti-human IgG (Fc specific)-FITC was from Sigma Aldrich (Munich, Germany), F(ab)’2 goat anti-mouse IgG-FITC, and F(ab)’2 goat anti-rabbit IgG-FITC was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Normal human serum (NHS) was used as a source of complement. Heat-inactivated serum (56°C, 30 min) served as negative control in all the experiments.

**siRNA molecules**

The siRNA molecules (BioSpring, Frankfurt, Germany) used in the study were blunt end, 19-mer double-stranded RNA oligo-nucleotides chemically stabilized by alternating 2’-O-methyl sugar modifications on both strands,54 where the unmodified
siRNA molecules used in the study were: siRNA control (random sequence)

5’ ugcagguaauagucca 3’ (sense)
5’ uugagcuauaaacucga 3’ (antisense)

siRNA anti-CD46

5’ gaggagaagacguaauug 3’ (sense)
5’ caaauagucucucucg 3’ (antisense)

siRNA anti-CD55

5’ gaggagucacgaaaaauug 3’ (sense)
5’ uacgagcacucugcuc 3’ (antisense)

siRNA anti-CD59

5’ gcaagaagccacuguaa 3’ (sense)
5’ uuaacagccucucucu 3’ (antisense)

Nucleotides with 2’-O-methyl modifications are underlined.

siRNA-transfection – amaxa electroporation

Raji and REH cells were transfected using the Cell Nucleofector® Kit V according to the manufacturers’ instructions (Axima, Cologne, Germany). After centrifugation, 2 × 10⁶ cells were resuspended in Nucleofector solution and 3 μg of each α-CD46, α-CD55, α-CD59 or control siRNA was added. For combined siRNA-transfection, 3 μg of each specific siRNA was used. For Raji cells, the Nucleofector program M-013 and for REH cells program W-001 was used. Mock experiments included Nucleofector solution alone. Cells were immediately transferred to pre-warmed culture medium and cultured for 3 d.

Flow cytometry

Tumor cells (1 × 10⁶) were pelleted, washed, and resuspended in 100 μL FACS-buffer (1% BSA, 0.1% NaN₃ in PBS). Cells were incubated with primary antibody or a corresponding isotype control (10 μg/mL). The cells were washed twice with FACS buffer, resuspended in 100 μL FACS buffer containing the respective FITC-conjugated secondary antibody and incubated in the dark for 30 min at 4°C. Finally, cells were washed three times in FACS buffer and fixed with 1% paraformaldehyde in PBS. Stained cells were analyzed by FACS Calibur using CellQuest Pro software (BD Biosciences, Heidelberg, Germany).

Complement-dependent cytotoxicity assay

Complement-dependent lysis of leukemia cells was analyzed by radioactive °⁵¹ Chromium release assay. Leukemia cells (1 × 10⁶) were labeled in 100 μL complete growth medium with 100 μCi °⁵¹ Cr (Hartmann Analytik, Braunschweig, Germany) for 2 h at 37°C. Cells were washed three times in assay medium (0.6 mM MgCl₂ and 0.1% BSA in RPMI without FCS) and adjusted to 2 × 10⁵ cells/mL in assay medium. Cells (10⁴ cells/50 μL) were pre-incubated for 30 min at 37°C with mAbs RTX or OFA or ALM (10 μg/mL). In experiments with CLL cells, mCRP specific non-complement activating neutralizing antibodies anti-CD46 (clone GB-24), anti-CD55 (clone Bric 110), and anti-CD59 (clone Bric 229) were applied at a concentration of 2 μg/mL to neutralize the respective inhibitors. TRX was used as control antibody. NHS (diluted 1:10 in assay medium) or heat-inactivated serum as control was added as complement source and incubated for 60 min at 37°C. To determine spontaneous °⁵¹ Cr release, NHS was replaced with test medium. Maximal °⁵¹ Cr release was determined by addition of 1% Triton®X-100 (Roche, Mannheim, Germany). Finally, the plates were centrifuged at 1200 rpm for 5 min and the radioactivity in supernatants was measured in a γ-counter (Wizard² 2470, PerkinElmer, MA, USA). The percentage of specific lysis of each sample was calculated according to the formula ((test release – spontaneous release)/(maximum release – spontaneous release)) × 100. All experiments were performed in triplicates.

C3 opsonization

Tumor cells (1 × 10⁵) were resuspended in 100 μL VBS-buffer (5 mM Sodium Barbital (pH 7.4), 0.15 mM CaCl₂, 1 mM MgCl₂, 150 mM NaCl; 0.1% BSA). To activate complement, cells were pre-incubated with RTX or OFA or ALM at a final concentration of 10 μg/mL for 30 min at 37°C. In experiments with CLL cells, mCRP specific non-complement activating neutralizing antibodies monoclonal mouse anti-human CD46 (clone GB-24) and monoclonal mouse anti-human CD55 (clone Bric 110) were applied at a concentration of 2 μg/mL to neutralize respective inhibitor expression. C8 depleted human serum (CompTech, Tyler, Texas, USA) was used for stimulation of Raji and REH cells (primary antibody: mouse monoclonal anti-iC3b / IgG; secondary antibody: FITC goat anti-mouse) and C3d in case of CLL cells to avoid cross-reactivity with mCRPs specific mouse antibodies (primary antibody: rabbit polyclonal anti-C3d / IgG; secondary antibody: FITC goat anti-rabbit).

Preparation of monocyte-derived macrophages

Isolation and cultivation of human monocytes/macrophages was done as described before. Briefly, PBLs were isolated from buffy coats by density gradient centrifugation. PBLs were subjected to CD14⁺ positive magnetic cell sorting using CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was determined by staining with FITC-labeled anti-CD14 antibody (ebiologic, Frankfurt, Germany) and cell purity of >90% was used for further experiments. Cells were resuspended in RPMI 1640-supplemented medium and cultured at a final concentration of 1 × 10⁶ cells/mL. The cell suspension was supplemented with IFNγ (1000 U/mL), or M-CSF (50 ng/mL) (Tebu Bio, Frankfurt, Germany), incubated at 37°C, 5% CO₂ for 6 d and subjected to further analysis. For phagocytosis experiments, cells were cultured on a cover slip with M-CSF (50 ng/mL).
ADCC and complement-dependent cellular cytotoxicity

ADCC and CDCC of tumor cells by PBLs or in vitro generated monocyte-derived macrophages, respectively, was analyzed by 51Cr release assay. Tumor cells were labeled with 51Cr and deposition of C3b/iC3b on tumor cells was performed as described above. PBLs or human monocyte-derived macrophages (see above) were added at an E:T ratio of 20:1 (PBLs) or 10:1 (macrophages) and cells were incubated at 37°C, 5% CO₂ for 4 h. Cells were centrifuged for 5 min at 1200 rpm and radioactivity in supernatants was measured in a γ-counter. Percentage specific release was calculated as ([(test release – spontaneous release)/(maximum release – spontaneous release)] × 100. All experiments were performed in triplicates.

Phagocytosis assay

CD14+ monocytes were cultured in the presence of 50 ng/mL M-CSF on coverslips as described above. On day 6, cells were stained with the green fluorescent dye CFSE at 1 μM (Molecular Probes) for 30 min, washed, and incubated in culture medium. CLL cells were stained with the lipophilic red fluorescent dye PKH26 at 1 μM (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, washed and resuspended in assay medium (0.6 mM MgCl₂ and 0.1% BSA in RPMI without FCS). Labeled cells were incubated with tumor-directed therapeutic mAbs and in some experiments, mCRP specific blocking antibodies anti-Cd46 (clone GB-24) and anti-CD55 (clone Bric 110) were applied at a concentration of 2 μg/mL. C8 depleted human serum was added to permit deposition of C3b/iC3b as described above. After 20 min, CLL cells were spun down and resuspended in assay medium. Opsonized CLL cells were then added to macrophages at a 1:1 ratio and incubated at 37°C for 2-3 h. After washing, macrophages were fixed with 2% paraformaldehyde and nuclei were stained with 1.5 μg/mL Hoechst 33342 dye (Life Technologies, Darmstadt, Germany) in 0.1% saponin/PBS buffer for 10 min. Samples were mounted on fluorescent mounting medium (Dako Cytomation, Hamburg, Germany) and fluorescence images were acquired using a Digital Fluorescence Microscope (Keyence, Neu-Isenburg, Germany) at ×20 magnification. Double positive macrophages with two nuclei (one large nucleus from macrophage and one or more smaller nuclei from CLL) were only taken into account for macrophages succeeded in phagocytosing CLL cells. Phagocytosis was evaluated by counting at least 400 cells for each experimental condition under fluorescent images from 10 different microscopy fields using the ImageJ processing and analysis software, and calculating the percentage of macrophages that engulfed at least one opsonized CLL cells with respect to total macrophages.

Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 6.0, first applying ANOVA tests and followed by Dunnett’s multiple comparisons test. Significance was accepted when p values were < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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