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Immunogenization of acute lymphoblastic leukemia cells with CpG oligodeoxynucleotides, IL-4 and CD40 ligand

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Für Sebastian und alle leukämiekranken Kinder
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACK</td>
<td>Ammonium-chloride-potassium lysing buffer</td>
</tr>
<tr>
<td>AIM-V</td>
<td>Adoptive Immunotherapy Media, defined serum free medium</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>BCP-ALL</td>
<td>B cell precursor-ALL</td>
</tr>
<tr>
<td>B-CLL</td>
<td>B cell chronic lymphatic leukemia</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4, found on the surface of T helper cells</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8, found on the surface of cytotoxic T cells</td>
</tr>
<tr>
<td>CD19</td>
<td>Cluster of differentiation 19, found on the surface of B cells</td>
</tr>
<tr>
<td>CD28</td>
<td>Cluster of differentiation 28, costimulatory molecule on T cells, binding to CD80</td>
</tr>
<tr>
<td>CD40</td>
<td>Cluster of differentiation 40, costimulatory molecule expressed on B cells</td>
</tr>
<tr>
<td>CD40L=CD154</td>
<td>Cluster of differentiation 40 Ligand, Member of TNF-superfamily, expressed on activated T cells, binds to CD40</td>
</tr>
<tr>
<td>CD54</td>
<td>Cluster of differentiation 54; intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>CD62L</td>
<td>Cluster of differentiation 62 Ligand; L-selectin, found on lymphocytes</td>
</tr>
<tr>
<td>CD80</td>
<td>Cluster of differentiation 80; B7-1, costimulatory molecule on B cells for T cell activation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>CD86</td>
<td>Cluster of differentiation 86; B7-2, costimulatory molecule on B cells for T cell activation</td>
</tr>
<tr>
<td>CD95</td>
<td>Cluster of differentiation 95; apoptosis antigen 1,</td>
</tr>
<tr>
<td>CD132</td>
<td>Common cytokine receptor γ-chain</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester, staining for proliferation assays</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie, unit for measuring radioactivity, named after Pierre Curie</td>
</tr>
<tr>
<td>CpG ODN</td>
<td>Cytosin-phosphate-guanosin oligodeoxynucleotide</td>
</tr>
<tr>
<td>CpG B</td>
<td>Class B CpG ODN, strong stimulator of B cells</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid, anticoagulant by binding calcium</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FasL</td>
<td>Ligand of CD95, member of tumor necrosis factor family, induces apoptosis</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC/SSC</td>
<td>Forward scatter/side scatter</td>
</tr>
<tr>
<td>GvHD</td>
<td>Graft versus Host Disease</td>
</tr>
<tr>
<td>GvL</td>
<td>Graft versus Leukemia</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplantation</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobuline</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1 = CD54</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen, binding to CD54=ICAM-1</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensities</td>
</tr>
<tr>
<td>MHC class I/II</td>
<td>Major histocompatibility complex class I or II</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------------</td>
<td>----------------------------------------------------------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NOD-SCID mice</td>
<td>Non-Obese Diabetic Severe Combined Immunodeficiency mice;</td>
</tr>
<tr>
<td>Ox40L</td>
<td>Ligand of CD134, expressed on a subtype of dendritic cells</td>
</tr>
<tr>
<td>panT cells</td>
<td>Mixed population of CD4 and CD8 positive T cells</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline without calcium and magnesium</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin-chlorophyll-protein complex</td>
</tr>
<tr>
<td>PerCPCy5.5</td>
<td>Peridinin-chlorophyll-protein complex (tandem with) cyanine dye 5.5</td>
</tr>
<tr>
<td>RPMI Medium</td>
<td>Medium for cell culture, developed by Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of means</td>
</tr>
<tr>
<td>TBP</td>
<td>TATTA box-binding protein</td>
</tr>
<tr>
<td>Th1/2 cell</td>
<td>T helper 1/2 cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Xeno ALL cells</td>
<td>ALL cells expanded in a NOD-SCID mouse xenograft model</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Acute lymphoblastic leukemia - epidemiology and basics

Leukemias are accountable for over 30% of all childhood malignancies [36]. Most of them are B cell precursor acute lymphoblastic leukemias (BCP-ALL). The overall prognosis is good, with a 5-year-survival rate of over 80% [42], [38]. This high curing rate, however, mostly represents the patients that are assigned to the so called standard risk group. High risk patients have a much worse outcome. And overall still up to a quarter of pediatric ALL patients in remission suffer from relapse, meaning once more intensive chemotherapy or hematopoietic stem cell transplantation, a much worse prognosis and limited treatment options [46]. A situation that shows the need for further investigation on treatment options, especially those targeting residual leukemic cells, is the occurrence of minimal-residual disease, which is considered to be the most predictive value for prognosis [7].

In patients aged between 15 and 29, leukemias represent only 6% of all cancers. Unfortunately, the prognosis becomes worse with increasing age. [37] Adult ALL patients more often suffer from early relapses, treatment-induced deaths and reach a 3-year-survival of only approximately 60%. Pediatric treatment regimes used in this age group resulted in slight improvement in outcome, but overall results are not satisfying [19]. Therefore, there is a need for more extended search for additional treatment options, especially for patients with poor prognosis.
1.2 Immunotherapy and characteristics of BCP-ALL cells

A research domain not yet fully exploited for fighting residual tumor cells is immunotherapy. It is performed either as active or passive immunization. Active immunization means on the one hand treating the patient with immunostimulating agents, on the other hand vaccinating the patient with tumor cell solutions or dendritic cells loaded with tumor antigen. In passive immunization pretreated T cells or NK cells are transferred or monoclonal antibodies targeting tumor antigen are given. Until now only monoclonal antibodies and donor-lymphocyte infusions after stem cell transplantation have shown curative capacity in clinical studies [6].

Many studies showed that the state of the immune system is related to tumor progression. Already in the 1700s, a relation between infections or inflammation and tumor progression was observed. In some studies certain cancer patients experienced tumor regression after they had acquired and recovered from bacterial infections [45]. More recent studies show that higher incidence rates of cancer are found in patients under immunosuppression after transplantation [41]. Furthermore, other authors report that infiltration of immune cells within certain resected tumor samples of colon cancer had a higher prognostic impact than tumor stage and nodal status [17]. In conclusion, these examples give a hint to the relation between high immune cell activity and less tumor progression and vice versa.

Immunotherapy of lymphoproliferative malignancies is of particular interest, due to the possibility not only to prime immune cells to fight the tumor, but also to be able to affect the tumor cells themselves, through their repertoire of receptors and surface molecules.

Like many other tumor cells, BCP-ALL cells are known to express low levels of costimulatory and antigen presenting molecules. This leads to the fact that they are poor T cell stimulators and are even able to induce anergy in naïve T cells [2], [4]. Simultaneously, BCP-ALL cells prevent themselves from NK cell killing through the low expression of MHC I molecules. These attributes enable leukemia cells to escape the control of potential anti-tumor cells in different ways and impede the profound development of an effective cellular anti-leukemic immune response.

Hence, the aim of some immunotherapeutic approaches is to find ways to enhance the immunogenicity of BCP-ALL cells, by increasing the expression of costimulatory
and surface molecules to reinforce intercellular communication and recognition. Although BCP-ALL cells show the escape mechanisms described above, they still express some cytokine and toll-like receptors (TLR) [40], offering the possibility to affect them with the help of compatible cytokines and TLR agonists.

1.3 Stimulators of normal and malignant B cells

1.3.1 Toll-like receptor 9 agonists and CpG oligodeoxynucleotides

Toll-like receptor 9 (TLR9) is one of the most abundant TLRs B cells express. Ligands of TLR9 are oligodeoxynucleotides, which contain motifs of unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides (CpG) that mimic bacterial or viral DNA fragments. In normal B cells ligation of CpG B to TLR9 leads to proliferation, cytokine production and upregulation of costimulatory molecules [29]. In malignant B cells, CpG B hardly induce any proliferation, but lead to a more pronounced immunogenic phenotype that is able to induce stronger reactions of the immune system than untreated malignant B cells [12], [23], [20]. Furthermore it seems likely that CpG do regulate expression of interleukin (IL)-2 family receptors. [11]

1.3.2 Cytokines of the interleukin-2 family

Some cytokine receptors of the IL-2 cytokine family can be detected on normal B cells, B cell chronic lymphoblastic leukemia (B CLL) cells and BCP-ALL cells. The IL-2 receptor family shares the common cytokine receptor γ-chain (CD132) and the additional private receptor chains are specific for the cytokines IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, respectively. IL-4 receptor, for example, is expressed on B cells. Ligation of its ligand IL-4, secreted by T helper cells, strongly activates the B cell by triggering MHC class II expression, immunoglobulin (Ig) class-switch and antibody secretion. Furthermore, some of the IL-2 family cytokines are said to synergize with each other, such as IL-4 and IL-21, which influence regulation of Ig class-switch [27].

1.3.3 CD40 - CD40 ligand

Looking at natural ways of cell communication, ligation of CD40 with CD154 (=CD40 ligand, CD40L) is a possibility of stimulating B cells in vitro. CD40L is expressed by activated T helper cells and binds to CD40, a costimulatory molecule expressed by B
cells. CD40 is a member of the tumor necrosis factor family and is induced by CpG B in B cells and B-CLL cells [22]. Ligation of CD40 by CD40L results in a strong B cell stimulatory signal and facilitates B cell maturation via the important second signal required for T cell activation [39]. In B cells, this signal leads to clonal expansion, antigen presentation, expression of costimulatory molecules, antibody secretion and cytokine production [39]. Furthermore, in patients with ALL it was observed that dysfunctional presentation of CD40L in bone marrow T cells leads to further disease progression, thought to be due to impeded cross talk between ALL and T cells [49], [2], [4].

1.4 Selected approaches for immunotherapy

Other groups show that treatment of human ALL in xenograft mouse models with CpG in vivo is successful. Leukemic burden is reduced, the mice stay in durable remission and experience ongoing immune-mediated protection against leukemic relapse [16], [43]. Also, some early clinical trials treating other malignancies found positive effects of the combination therapy of TLR9 agonists with chemotherapy [28]. Although these results appear promising, none could be translated into therapeutic treatment of patients with ALL so far.

Unfortunately, CpG has the characteristic to bind to serum proteins in relevant amounts, leading to the fact that doses used in in-vivo-approaches can result in levels too low to induce relevant changes in the immunogenic phenotype of leukemia cells [21]. Additionally, in-vitro-treatment with CpG alone doesn’t always succeed in initiating a reliable immune response against stimulated leukemia cells. Therefore, combination of differential ways of stimulating BCP-ALL cells appears promising [24], especially in in-vitro-stimulation, where higher doses of CpG can be used without inducing dangerous adverse effects.

1.5 Aims and questions

This work is based on the concept of treating BCP-ALL cells in vitro to enhance their immunogenicity in a way that forces them to induce an active cellular immune response.

For this purpose, the B cell stimulators CpG B, IL-4 and soluble CD40L alone and in combination were evaluated. To address this aim we used BCP-ALL cell lines, BCP-ALL cells that were expanded in a murine xenograft model and primary patient
samples. Questions to be answered in this work are:

- Are CpG B, IL-4 and soluble CD40L potent upregulators of costimulatory and antigen-presenting molecules on BCP-ALL cells?

- Is the combination of the stimulators mentioned above more potent than each reagent alone?

- Can such stimulated BCP-ALL cells induce physiologic reactions, like T cell proliferation?

- Are such pretreated BCP-ALL cells efficient stimulators in the generation of anti-leukemic cytotoxic T cells (CTL)?

- Can CTL generated with stimulated BCP-ALL cells even specifically kill untreated BCP-ALL cells?

- Do CTL generated with such pretreated BCP-ALL cells also specifically kill in autologous in vitro approaches?
2 Materials and methods

2.1 Samples, cell lines and cell culture

B cell precursor (BCP)-ALL cell lines KOPN-8, MHH-CALL-2 (MHH), Nalm-6 and REH were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), RS4;11 from American Type Culture Collection (Manassas, VA). Cell line 018Z was generously provided by Dr L.H. Meyer (Ulm University Children’s Hospital, Ulm, Germany). All cell lines except for MHH and 018Z were cultured in RPMI 10% medium (RPMI 1640 GIBCO, Invitrogen GmbH, Karlsruhe, Germany) with 10% fetal bovine serum (FBS, Invitrogen GmbH, Karlsruhe, Germany) and each 1% L-Glutamine (Invitrogen GmbH), HEPES buffer (Biochrom AG, Berlin, Germany) and Sodium-Pyruvate (Biochrom AG) (complete medium). MHH and 018Z were cultured in RPMI medium with the same additives but 20% FBS. Fresh and cryopreserved BCP-ALL cells that had been expanded in NOD/SCID mice from patient samples in a xenograft model (Xeno BCP-ALL) were kindly provided by Dr L.H. Meyer (Ulm University Children’s Hospital, Ulm, Germany, [33]). For functional assays these cells were cultured in the RPMI medium with 20% FBS as above.

To obtain peripheral blood mononuclear cells (PBMC) 5 - 50 ml of peripheral blood was taken with informed consent from healthy individuals or BCP-ALL patients in remission that had finished treatment according to the ALL BFM-95 or -2000 protocols. For some experiments, PBMC were isolated from buffy coats from the DRK Blood Bank in Ulm (German Red Cross). Blood was collected either in standard 10 ml citrate tubes (Sarstedt, Nuembrecht, Germany) or in 50 ml syringes (BD, Drogheda, Ireland), which were coated with 250 µl sodium-heparin containing 250 I.U. (B. Braun Melsungen AG, Melsungen, Germany). Blood or buffy coat samples were immediately diluted 1:2 with phosphate buffered saline without calcium and magnesium (PBS, Biochrom AG, Berlin, Germany). In 50 ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA) 20 ml of the diluted blood were layered on 15 ml Biocoll Separating Solution (Biochrom AG, Berlin, Germany) and filled up with PBS for density gradient centrifugation with 1000 × g at 20°C for 15 minutes with brakes off. Then the white
rings containing PBMC were harvested into 50 ml tubes and washed with PBS (400 x g, 10 minutes, 20°C). Lysis of erythrocytes was performed by 7 minutes incubation with 5-10 ml of Ammonium-chloride-potassium (ACK) lysing buffer (0,15 M NH₄Cl, 1 mM KHCO₃, 0,1 mM ethylenediaminetetraacetic acid (EDTA, Carl Roth, Karlsruhe, Germany), pH 7,3) and ended by washing the cells twice with PBS. After counting in a Neubaur counting-chamber (Laboroptik, Friedrichdorf, Germany) with trypan blue (Sigma-Aldrich, Steinheim, Germany), cells were cultured in either AIM-V (Adaptive Immunotherapy Media) media (Gibco BRL, Grand Island, NY, USA) or complete RPMI medium with 10% FBS.

2.2 Pretreatment of BCP-ALL cells

For every functional assay we pretreated the BCP-ALL cells, both cell lines and xenocytes with different combinations of the following immunostimulators: CpG oligodeoxynucleotides with the specific sequence 5'-TCG TCG TTTCGT CGT TTT GTC GTT-3’ (Coley Pharmaceutical Group, Ottawa, Canada) (CpG ODN, class B: 10104), interleukins (IL)-4 (Strathmann Biotec GmbH & Co KG, Hamburg, Germany) and IL-21 (BioSource, Camarillo, CA), soluble CD40 ligand in combination with enhancer for ligands (both from Alexis Biochemicals, Enzo Life Sciences GmbH, Lörrach, Germany). BCP-ALL cells were cultured for two days in the previously described adequate RPMI medium at a density of 5x10⁶ to 1x10⁷ cells/ml and pretreated with either medium, CpG ODN in final concentration (fc) of 2.5 µg/ml, IL-4 (fc: 400 U/ml), IL-21 (fc: 100 ng/ml), soluble cluster of differentiation (CD) 40 ligand in a final concentration of 1 µg/ml with enhancer for ligands (fc: 1 µg/ml) or combinations. After 24 hours cells were resuspended with medium and the same reagents in the above mentioned concentrations. After 48 hours of incubation we harvested the ALL cells and either analysed them immediately or used them in functional assays.

2.3 Flow cytometry

To define phenotype and measure changes in surface molecule expression after stimulation, flow cytometry was performed either on a FACS Scan or on a LSR II (both Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Results were expressed as median fluorescence intensities (MFI) and analyzed using FlowJo software version 8.7.1 (Tree Star, Stanford, CA, USA). Cells to be analyzed were harvested into fluorescence activated cell sorting (FACS) tubes and washed with 2 ml PBS (300 x g,
7 minutes, 20°C). Supernatants were discarded, cell pellets resuspended and stained by adding 3 µl of each indicated antibody. After 10 minutes of incubation at room temperature and dark, samples were washed again with 2 ml PBS (300 x g, 7 minutes, 20°C) and fluorescence was measured immediately.

For gating at precursor B-ALL cells we used conjugated antibodies against CD19 and CD10, the latter being expressed on precursor, but not mature B cells. To define T cells we gated on CD4 or CD8 positive, CD19 negative cells. To analyze the status of stimulation of the ALL cells after pretreatment we stained with conjugated antibodies against surface molecules and antigen presenting molecules, namely CD40, CD54, CD86, CD80, MHC class I, MHC class II, CD95, FasL, CD62L and Ox40L. The antibodies against these molecules were conjugated with FITC, PE, PE-Cy5, PerCP, PerCP-Cy5.5, APC and AmCyan (Becton Dickinson, Heidelberg, Germany). Because of variability in basal expression of these markers on the leukemia cells, we defined increased expression as relative value, which is expression of stimulated cells compared to non-stimulated cultured cells.

2.4 Toll-like receptor 9 real time polymerase chain reaction (PCR)

For quantitative analysis of toll-like receptor (TLR) 9 messenger ribonucleic acid (mRNA) expression, the BCP-ALL cell lines RS4;11, KOPN-8 and 018Z were incubated with CpG (2.5 µg/ml) in the presence or absence of CD40L (1 µg/ml) and IL-4 (400 U/ml) for 48 h. Untreated cells served as control. According to the manufacturer’s protocol, total RNA was extracted from 2x10⁶ BCP-ALL cells per sample using an RNeasy mini kit (Qiagen, Hilden, Germany). Genomic deoxyribonucleic acid (DNA) was digested with RNase-free DNAse I set (Qiagen) and cDNA synthesis of 1 µg total RNA was performed using RT Superscript III (Invitrogen, Carlsbad, CA, USA). Primers for TLR9 mRNA were as follows: forward 5’-gccagaccctctggagaa-3’, reverse 5’-agaacctccaagagagcgttg-3’. PCR products were analyzed with a Light Cycler 2 (Roche, Mannheim, Germany) using the SYBR Green PCR kit (Qiagen). PCR signals were normalized to average expression of the housekeeping genes TATA box-binding protein (TBP) and RPL-32 by subtracting the mean cycle threshold (Ct) value of the reference gene from the Ct value of the TLR9 gene for each sample (ΔCT). To compare each sample to the untreated control, ΔΔCT values were calculated by subtraction of ΔCT values of untreated from treated samples. Final values were plotted as 2-ΔΔCT (PE
2.5 Mixed lymphocyte reaction, proliferation assay

To investigate functional aspects in conditions that are comparable to physiological conditions, we performed mixed lymphocyte assays (MLR = mixed lymphocyte reaction) in several ratios between different cell types. Pre-treated leukemia cells acted as stimulators for PBMC that were isolated from peripheral blood or buffy coats as described before or panT cells (CD4 and CD8 positive T cells) isolated using a Pan T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany).

In order to observe proliferation, we performed 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) staining or ³H-thymidine-assay.

For CFSE staining isolated PBMC were washed once in PBS (400 x g, 10 minutes, 20°C) and counted in a Neubauer chamber. 3x10^7 cells were resuspended in 1.2 ml PBS and CFSE (Molecular Probes, Eugene, OR, USA) was added with a final concentration of 5 µM. Cells were incubated for 10 minutes at 37°C in the incubator, blocked with 10 ml cold AIM-V medium and washed three times with 30 ml PBS (400 x g, 10 minutes, 20°C). Allogeneic CFSE-labeled PBMC were then cocultured as before with pretreated leukemia cells in different ratios. After five days cells were harvested, stained with anti-CD19 PE, anti-CD8 PerCP, anti-CD4 APC conjugated antibodies (Becton Dickinson, Heidelberg, Germany) as described above and analyzed by gating on viable CFSE low CD4+ and CD8+ T cells excluding CD19+ BCP-ALL cells using LSR II and FlowJo software.

For ³H-thymidine-assay, PBMC were cultured in RPMI medium (5x10^5 ml) directly after isolation together with either pretreated or non-treated leukemia cells in PBMC:ALL ratios 1:5, 1:10, 1:100, 1:1000. After 4 days in culture 1 µCi/well ³H-thymidine was added and incubated for 18h at 37°C and 5% CO₂. Then cell interaction was stopped by freezing for 4 hours in -80°C and cells were harvested with a cell harvester (Inotech Biosystems International Inc., Dietikon, Switzerland) and analyzed in a Top CountNXT™ counter (Perkin Elmer, Rodgau-Jügesheim, Germany).

2.6 CTL culture and chromium-release assay

Alloreactive or syngeneic cytotoxic T cells (CTL) were generated by incubating Biocoll-isolated PBMC from healthy donors (first week 2x10^6/ml, following weeks 1x10^6/ml)
with either untreated or pretreated BCP-ALL cells as stimulators at an effector : stimulator (E:S) ratio of 10:1 in RPMI 10% as described above, but without HEPES buffer. Restimulation with BCP-ALL cells was performed weekly at the same E:S ratio. In case of high amounts of dead cells (>60%) in flow cytometry analysis, detected by gating on viable cells in forward scatter/side scatter (FSC/SSC), dead cells were removed by Biocoll-density gradient isolation prior to stimulation. At day 4 after each stimulation and with every restimulation 30 U/ml recombinant human Interleukin 2 (IL-2) (PeproTech GmbH, Hamburg, Germany) was added. In order to suppress proliferation, BCP-ALL cell lines were treated with 50 µg/ml mitomycin C (SERVA Electrophoresis GmbH, Heidelberg, Germany) before addition, whereas primary Xeno BCP-ALL cells, with very limited survival in culture, were used as stimulators without mitomycin C treatment.

To test cytotoxicity, at day 6 after PBMC stimulation, 3x10^6 untreated BCP-ALL cell line cells, as target cells, were labeled with 150 µCi Na^51^Cr^4+ (Amersham-Buchler, Braunschweig, Germany) for 1-1.5 h. To 5x10^3 target cells effector cells were added at an effector : target (E:T) ratio of 50:1, serially diluted and incubated for 4 hours at 37°C and 5% CO_2. 50 µl of supernatant was harvested for ^51^Cr-release in triplicates in a Top CountNXT™ counter (Perkin Elmer, Rodgau-Jügesheim, Germany). Maximum release was determined by incubation of target cells in 100 µl 10% sodium dodecyl sulfate (SDS), for spontaneous release they were incubated in medium. The percentage of specific lysis was calculated as % specific release = (experimental release - spontaneous release) / (maximum release - spontaneous release) x 100.

2.7 Cytometric bead array

For cytokine detection and quantification we used supernatants from BCP-ALL cell stimulations. BCP-ALL cells were incubated in complete medium with stimulation reagents as described above for 48h. Supernatants were harvested, frozen at -80°C and analyzed using a sandwich Bio-Plex cytokine immunoassay following the manufacturer’s specifications (Bio-Rad, Hercules, CA, USA). This assay permits simultaneous detection of IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IFN-γ and TNF-α. All incubations were carried out at 24°C. Briefly, the anti-cytokine bead solution was diluted and 50 µl placed on 96-well Durapore membrane plates (Millipore, Billerica, MA, USA). After washing by vacuum filtration, 50 µl of undiluted supernatants and standards were placed into wells and incubated for 30 minutes on a shaker. After washing, 25 µl detection antibody was added and incubated for another 30 minutes while shaking at
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300 rpm. After washing, streptavidin-PE was added for 10 minutes. Then, plates were washed, resuspended in assay buffer, read on a plate reader (Luminex, Riverside, CA, USA) and analyzed using Bio-Plex Manager software (Bio-Rad, Hercules, CA, USA).

2.8 Statistics

Data are expressed as means, aberrations as standard error of mean (SEM). To determine statistical differences between the means of two data columns, the paired two-tailed student’s t-test was used and carried out in Excel (Microsoft Corporation, USA). P-values were corrected using the Bonferroni method where applicable. Results were considered significant with p-values < 0.05.
3 Results

3.1 Enhancement of surface molecule expression

The following chapter describes ways to induce an immunogenic phenotype in BCP-ALL cells, beginning with basal expression of surface molecules prior to stimulation to differences in response between different BCP-ALL cell types. Our aim was to enable recognition and eventually demolition of these malignant tumor cells by other immune cells by induction of an immunogenic phenotype.

3.1.1 Basal expression of surface molecules on BCP-ALL cells is highly variable

We worked with six different BCP-ALL cell lines, primary BCP-ALL cells and Xeno BCP-ALL cells expanded in a NOD/SCID mouse xenograft model. We assumed that differences in basal surface molecule expression may result in different responsiveness of the BCP-ALL cells to stimulation. To measure basal expression untreated cells were stained for the costimulatory molecules CD40 and CD86, intercellular adhesion molecule CD54 and the antigen presenting molecules MHC class I and class II, followed by flow cytometry analysis.

As figure 1 shows, basal expression of all screened molecules was highly variable among the different types of BCP-ALL cells. We observed a relatively high MHC class I and II expression, with the highest values in primary BCP-ALL, followed by Xeno BCP-ALL. MHC expression in BCP-ALL cell lines was moderate, with the lowest expression in 018Z. All BCP-ALL cell types showed low, but detectable CD54 expression, but very weak CD86 and CD40 expression.
Results

Figure 1: Untreated B cell precursor-acute lymphoblastic leukemia cells (BCP-ALL cells) show highly variable basal surface molecule expression. We examined BCP-ALL cell lines (RS4;11, KOPN-8, O18Z, MHH-CALL-2, Nalm-6, Reh), primary BCP-ALL cells, which were derived directly from patients, and BCP-ALL cells that had been expanded in nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice from patient samples in a xenograft model (Xeno BCP-ALL cells). Cells were stained for cluster of differentiation (CD) 40, CD54, CD86, major histocompatibility complex class I (MHC class I) and MHC class II and analyzed by flow cytometry. Bar graphs represent median fluorescence intensities (MFI) of the tested surface molecules from at least 3 independent experiments. Error bars indicate standard error of means.

3.1.2 Combination of CpG B, IL-2 family cytokines and CD40 ligand is more effective in inducing an immunogenic phenotype in BCP-ALL cell lines than treatment with CpG B alone

CpG B has been reported to induce distinct changes in the phenotype of normal B cells, as well as in malignant B cells. The effect is shown in different B cell malignancies, like lymphomas, chronic lymphatic leukemia (B-CLL) and B cell precursor acute lymphoblastic leukemia. This study investigates the effect of CpG B in combination with the B cell stimulatory agents IL-2 family cytokines and CD40 ligand on BCP-ALL cells compared to CpG B alone. To evaluate phenotypic changes after stimulation we treated different types of BCP-ALL cells with CpG B, IL-4, IL-21 or CD40L as single agents and with the two triple combinations CpG B plus IL-4 plus CD40L and CpG B plus IL-21 plus CD40L. Changes in phenotype induced by IL-4, IL-21 and CD40L as single agents were rather subtle, whereas CpG B alone induced moderate upregulation of the measured surface molecules. However, the triple combinations of IL-4, IL-21 and
CD40L with CpG B did induce strong synergistic enhancement of surface molecule expression. Figure 2 A demonstrates these effects as representative histograms of CD54 upregulation in the cell line RS4;11. Bar graphs in figure 2 B show average relative expression for the cell lines RS4;11 and KOPN-8. Levels of CD54 expression rose from a moderate level with CpG B alone, to a slightly higher level with one additional reagent and to a significantly higher level with triple combination treatment. In addition, figure 2 B shows a significant difference between the two triple combinations, with higher results for CpG B with IL-4 and CD40L.

In addition to CD54, we also tested the effect on the costimulatory molecules CD40 and CD86 and antigen presenting molecules MHC class I and class II on all six cell lines. We could divide the tested cell lines into two groups, the group of poor responding cell lines, MHH-CALL-2, Nalm-6 and REH, and stronger reacting ones, 018Z, RS4;11 and KOPN-8 (figure 3). The highest upregulation after stimulation could be observed with KOPN-8 and RS4;11 for the molecules CD40 and CD54. Upregulation was up to 80% after treatment with CpG B, IL-4 and CD40L compared to medium for KOPN-8 and up to 70% for RS4;11, which was significantly higher than upregulation after treatment with CpG B as single agent. Also in the poor reacting group, cell line MHH-CALL-2 responded with significant, but lower upregulation of CD40 and CD54 after treatment with CpG B, IL-4 and CD40L. Expression of CD86, MHC class I and class II was upregulated to a small extend in all cell lines. The exceptions were RS4;11 and 018Z. RS4;11 upregulated MHC class II up to 20% after treatment with CpG B, IL-4 and CD40L and also showed significant differences between single and combination treatment. Cell line 018Z showed changes in all tested markers, but no significant differences between the triple combination treatment and CpG B alone. For further studies of functional aspects, we decided to concentrate on the moderate and strong responding cell lines.
Results

Figure 2: Combination of cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) with cluster of differentiation (CD) 40 ligand (CD40L) and interleukin (IL-) 2 family cytokines results in enhanced upregulation of immunological crosstalk molecules. B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells were pre-treated over 48 hours with CpG B alone (2.5 µg/ml), or in combination with IL-4 (400 U/ml) and either IL-21 (100 ng/ml) or CD40L (1 µg/ml) along with an enhancer for ligands (1 µg/ml). Then, cells were harvested and analyzed by flow cytometry. A) Histograms show upregulation of CD54 on the cell line RS4;11 after combination treatment compared with upregulation after single agent treatment. Data are representative for six independent experiments with similar results. Dashed lines represent median fluorescence intensity of CD54 of medium control, grey areas show the isotype control. B) Bar graphs indicate average relative expression of CD54 on RS4;11 and KOPN-8 cells as median fluorescence intensity (MFI) after different treatment combinations in at least three independent experiments. ** Indicates p<0.01, * indicates p<0.05. Error bars in B indicate standard error of means.

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Results

Figure 3: Stimulation of B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells differentiates cell lines into a good and a poor responding group. As described before BCP-ALL cell line cells were pretreated over 48 hours with cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) alone (2.5 \( \mu \)g/ml), or in combination with interleukin- (IL-) 4 (400 U/ml) and either IL-21 (100 ng/ml) or CD40 ligand (1 \( \mu \)g/ml) along with an enhancer for ligands (1 \( \mu \)g/ml). Then, cells were harvested and analyzed by flow cytometry. Bar graphs represent mean fluorescence intensity (MFI), indicating increase of surface molecule expression compared to medium in six BCP-ALL cell lines. Data are from at least three independent experiments for each cell line. * Indicates p<0.05 as significant differences between treatment with CpG B alone and combination treatment. Note different y-axis scaling. Error bars indicate standard error of means.
3.1.3 Stimulation with CpG B, IL-2 family cytokines and CD40L induces significant increase in surface molecule expression in Xeno BCP-ALL and primary BCP-ALL cells

Although overall upregulation of markers of immunogenicity in cell lines was heterogeneously, the results could be confirmed in exemplarily tested human primary BCP-ALL cells and Xeno BCP-ALL cells, human BCP-ALL cells that were expanded in NOD/SCID mice (figure 4). In those primary or close to primary cells, it was possible to enhance the expression of immunogenic surface molecules to moderately high values. The highest increases of expression appeared, similar to the results in cell lines, for CD54 and CD40, but also for MHC class II. Combination treatment with CpG B, IL-4 and CD40L was significantly more effective than CpG B, IL-4 and IL-21 or CpG B alone. In Xeno BCP-ALL cells, upregulation of CD54 and MHC class II was significantly superior with CpG B, IL-4, CD40L combination treatment compared to CpG B alone. In contrast, in primary BCP-ALL cells treated with CpG B, IL-4 and CD40L upregulation of CD40, but not of CD54, was significantly higher than with CpG B alone. Also, CD54 expression did not differ after treatment with the two triple combinations. In contrast to tested BCP-ALL cell lines, primary BCP-ALL cells responded to stimulation with upregulation of CD86. Upregulation was only 16% increase of positive cells, but also displayed a significant advantage of triple combination treatment with CD40L compared to CpG B alone.
**Results**

Figure 4: Stimulation of primary B cell precursor-acute lymphoblastic leukemia cells (BCP-ALL cells) and Xeno BCP-ALL cells with the combination of cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) with interleukin (IL-)2 family cytokines or cluster of differentiation (CD) 40 ligand (CD40L) leads to enhanced upregulation of surface molecules compared to CpG B alone. BCP-ALL cells were incubated with CpG B alone (2.5 µg/ml), or CpG B (2.5 µg/ml) in combination with IL-4 (400 U/ml) and either IL-21 (100 ng/ml) or CD40L (1 µg/ml) along with an enhancer for ligands (1 µg/ml) as indicated by different colours for 48 hours. Afterwards cells were harvested, washed and analyzed by flow cytometry. Bar graphs indicate average percentage of upregulation of CD40, CD54, CD86 and major histocompatibility complex (MHC) class I and II compared to medium control, shown as median fluorescence intensity (MFI). Presented data are from at least four independent experiments for primary BCP ALL cells and from at least three independent experiments for Xeno BCP-ALL cells. * indicates p<0.05 showing significant differences between treatment with CpG B alone and combination treatment. Note different y-axis scaling. Error bars indicate standard error of means.
3.1.4 IL-21 decreases viability of BCP-ALL cell line

In some BCP-ALL samples we found a consistent loss of viable cells. This loss exceeded the usual amount of dead cells that occurred to a small extent after in vitro treatment. After further testing the cytokine IL-21 was identified as responsible agent for the cell death. Since our ultimate goal was to use the stimulated BCP-ALL cells as stimulator cells for T cells to raise a potent anti-leukemic response, we intended to maintain stimulated viable BCP-ALL cells. However, IL-21, that was part of one of the triple combinations with CpG B and IL-4, decreased the viability of one cell line, RS4;11, to 36% living cells alone and to 7-14% in combination with CpG B (figure 5). This, along with the overall weaker response to the triple combination CpG B + IL-4 + IL-21, compared to CpG B + IL-4 + CD40L, led us to focus on treatment with CpG B, IL-4 and CD40L.

Figure 5: Interleukin- (IL-) 21 leads to higher cell death in B cell precursor-acute lymphoblastic leukemia (BCP-ALL) cell line RS4;11. Cells were incubated with the following agents in indicated combinations, cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) (2.5 µg/ml), IL-4 (400 U/ml), IL-21 (100 ng/ml) or cluster of differentiation (CD) 40 ligand (CD40L) (1 µg/ml) along with an enhancer for ligands (1 µg/ml) for 48 hours. Viability was determined by morphology, using the flow cytometric criteria for morphology in forward scatter and side scatter (FSC,SSC). Bar graphs represent average of viable cells from at least three independent experiments, error bars indicate standard error of means. ** indicates p < 0.01.
3.2 Higher amount of TLR9 mRNA is transcripted after treatment with IL-4, CD40L and CpG B.

It has been shown that B cells, including malignant B cells, express TLR9 and that CpG B is a potent TLR agonist. To investigate the mechanism of the higher stimulatory capacity of combined treatment of CpG B with IL-4 and CD40L, we tested three BCP-ALL cell lines for TLR9 mRNA expression after 48 hours of incubation with CpG B alone, IL-4 plus CD40L and CpG B plus IL-4 plus CD40L compared to medium control.

Figure 6: Induction of transcription of Toll-like receptor 9 (TLR9) messenger RNA (ribonucleic acid) in B cell precursor-acute lymphoblastic leukemia cells (BCP-ALL cells) through treatment with interleukin-4 (IL-4) and cluster of differentiation (CD) 40 Ligand (CD40L). Reverse transcription polymerase chain reaction (PCR) was done on the three BCP-ALL cell lines 018Z, RS4;11 and KOPN-8 for quantitative analysis. After 48 hours of incubation with IL-4 (400 U/ml), CD40L (1 µg/ml) along with an enhancer for ligands (1 µg/ml) plus or minus cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) (2.5 µg/ml) total RNA was extracted from $2 \times 10^6$ BCP-ALL cells per sample. As control served untreated cells of the same cell line. PCR signals were normalized to average expression of the housekeeping genes TATTA box-binding protein (TBP) and RPL-32 for each sample ($\Delta \Delta C_T$). To compare each sample to the untreated control, $\Delta \Delta C_T$ values were calculated by subtraction of $\Delta C_T$ values of untreated from treated samples. Final values were plotted as $2^{-\Delta \Delta C_T}$. Data results from three (018Z), six (RS4;11) and four (KOPN-8) independent experiments. Error bars indicate standard error of means. * indicates $p<0.05$, ** indicates $p<0.01$.

In two of three cell lines, treatment with IL-4 and CD40L increased the amount of TLR9 mRNA to a significantly higher level than CpG B alone or medium control. Hence, IL-4 in combination with CD40L seems to enhance the transcription of TLR9 mRNA.
A higher amount of mRNA leads to higher expression of TLR9 and thus extends the chance for CpG B to bind and lead to stimulation of the target cell (figure 6).

3.3 Induction of T cell proliferation

Next, we investigated, whether treatment of BCP-ALL cells with CpG B, IL-4 and CD40L holds functional relevance. Based on the assumption that by stimulation naturally poor immunogenic BCP-ALL cells are skewed to higher immunogenicity, they should be enabled to interact with other immune cells. An efficient anti-tumor immune response relies on T cell interaction, crucial effector cells of the immune system. As a first step we monitored the effect of differentially treated BCP-ALL cells on proliferation in allogeneic T cells. Under physiological conditions in the human body, antigen presenting cells, like B cells, induce proliferation in T cells that recognize the presented self or foreign antigen on MHC class I or II. But this only can lead to stimulation when enough costimulation takes place. Induction of proliferation has the aim to multiply effector cells that are specific for the presented antigen and thus to facilitate an effective immune response.

3.3.1 Combination treatment with CpG B, IL-4 and CD40L

proves superior to CpG B alone in BCP-ALL cells in their capacity to induce T cell proliferation

To investigate this aspect, we pretreated Xeno BCP-ALL cells with CpG B alone, CpG B + IL-4 + IL-21 or CpG B + IL-4 + CD40L for 48 hours. After thorough washing, the cells were coincubated with freshly isolated PBMC from healthy allogeneic donors. We analyzed T cell proliferation with two different methods, either with CFSE-staining (figure 7) or with $^3$H-Thymidine incorporation (figure 8). Advantage of CFSE-staining is the possibility to surely determine the proliferation as T cell proliferation by FACS-analysis.
**Results**

**Figure 7**: B cell precursor-acute lymphoblastic leukemia cells (BCP-ALL cells) pre-treated with the combination of cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B), interleukin- (IL-)4 and cluster of differentiation (CD) 40 Ligand (CD40L) prove superior to pretreatment with CpG B alone in the capacity to induce T cell proliferation. Xenocraft BCP-ALL cells were incubated with CpG B (2.5 µg/ml), IL-4 (400 U/ml) and either IL-21 (100 ng/ml) or CD40L (1 µg/ml) along with an enhancer for ligands (1 µg/ml) in given combinations for 48 hours. Afterwards cells were harvested, washed thoroughly and cocultured with freshly isolated and previously carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained peripheral blood mononuclear cells (PBMC) in a PBMC:ALL ratio of 10:1. After 5 days cells were harvested, stained and analysed by flow cytometry. Proliferated T cells were gated as CFSE-low, CD19-, CD4+ or CD8+ cells. Side scatter (SSC) is shown on y-axis. 6A shows representative histograms for CD4+ and CD8+ T cells in each pretreatment combination. As negative control served untreated PBMC, as positive control CD3/CD28 stimulated T cells. 6B: Bar graphs represent percentage of T cell proliferation as average of seven independent experiments with six different BCP-ALL donors. * indicates p<0.05, ** indicates p<0.01. Error bars indicate standard error of means.
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CFSE-low cells were gated for CD19-, CD4+ or CD8+ cells. Similar to phenotypic alteration described above, combination of CpG B with IL-4 and CD40L proved superior in enabling BCP-ALL cells to induce T cell proliferation. Xeno BCP-ALL cells pretreated with CpG B + IL-4 + CD40L induced proliferation in 30% of CD4+ T cells and in 16% of CD8+ T cells. Adding CD40L to CpG B + IL-4 was tendentially more effective than adding IL-21. Untreated Xeno BCP-ALL cells by contrast only induced minimal T cell proliferation. This difference was significant for both cell populations, CD4+ and CD8+ T cells. As indicated in figure 6A and B, particularly CD8+ T cell proliferation was significantly higher after triple combination pretreatment with CD40L than CpG B single treatment. These results were confirmed with three exemplary experiments with $^3$H-Thymidine incorporation, where we detected similar differences between the effect of medium and combination pretreated Xeno BCP-ALL cells (figure 8). Furthermore, the T cell proliferation induced by BCP-ALL cells pretreated with CpG B, IL-4 and CD40L showed a dependency on the ratio of BCP-ALL cells to coincubated PBMC.

Figure 8: T cell proliferation is induced by cocultured pretreated B cell precursor-acute lymphoblastic leukemia cells (BCP-ALL cells). As stimulators BCP-ALL cell lines RS4;11, KOPN-8 and Xeno BCP-ALL cells were treated with only medium or cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) (2.5 µg/ml), IL-4 (400 U/ml) and CD40L (1 µg/ml) along with an enhancer for ligands (1 µg/ml) for 48 hours and after washing, cocultured with freshly isolated peripheral blood mononuclear cells (PBMC) for four days in PBMC:ALL ratios 1:5, 1:10, 1:100, 1:1000. For analysis $^3$H-Thymidine (5 µCi/ml) was added and incubated for 18 hours. After stopping the interaction by freezing for four hours, cells were harvested and analyzed. Bar graphs show exemplary results for each ALL type as counts per minute (CPM) of triplets of proliferated T cells after coculture with pretreated or untreated Xeno BCP-ALL cells or BCP-ALL cell lines KOPN-8 and RS4;11. Error bars indicate standard error of means of experimental triplicates.
3.4 Stimulated BCP-ALL cells produce proinflammatory cytokines

A way of communication and coordination between cells of the immune system is secretion of cytokines. As treatment of BCP-ALL cells with CpG B, IL-4 and CD40L leads to enhancement of immunogenicity, the next step was to characterize the secreted cytokines.

![Figure 9: B cell precursor-acute lymphoblastic leukemia cells (BCP-ALL cells) produce pro-inflammatory cytokines interleukin-12 (IL-12) and tumor-necrosis-factor α (TNF-α) after stimulation with the combination of cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B), interleukin-4 (IL-4) and cluster of differentiation (CD) 40 Ligand (CD40L). BCP-ALL cells were incubated with CpG B (2.5 µg/ml), IL-4 (400 U/ml) and CD40L (1 µg/ml) along with an enhancer for ligands (1 µg/ml) in different combinations or alone for 48 hours. Cytometric bead assay was performed with the supernatants to detect IL-12, TNF-α, IL-6 and IL-10. Bar graphs represent average concentrations of the indicated cytokine of four independent experiments. * indicates p < 0.05. Error bars indicate standard error of means.]

We could show that treatment with the triple combination of CpG B, IL-4 and CD40L induces secretion of IL-12 and tumor necrosis factor α (TNF-α) in relevant amounts. As demonstrated in figure 9 stimulation of BCP-ALL cells with CpG B alone or IL-4 and CD40L in combination does not lead to increased secretion of the tested cytokines, but combination of all reagents significantly increased the cytokine secretion. The other tested cytokines showed no significant increase through stimulation.
3.5 Induction of cytotoxicity in CTL against untreated leukemia target cells

Apart from activation and expansion of T cells, an effective anti-tumor immune response also needs cells that are able to destroy the malignant target cell, either by phagocytosis or by lysis. Cytotoxic T lymphocytes are cells of the immune system able to lyse virus-infected or tumor cells in a specific manner, when they are stimulated the right way. To measure if our treatment, on top of changes in surface molecule expression, is able to lead to effective interactions between BCP-ALL cells and their immunologic counterparts CTL, we raised allogeneic CTL from PBMC. They were raised with differentially pretreated BCP-ALL cells and we used untreated BCP-ALL cells as targets to test them.

3.5.1 CTL raised with combination-treated stimulator

BCP-ALL cells exert a higher specific lysis compared to CTL of CpG B-only-pretreated stimulator cells

To compare the effect of differentially treated stimulator BCP-ALL cells, we raised in parallel three CTL lines from the same PBMC donor, CTL against untreated BCP-ALL cell lines KOPN-8 and RS4;11, CTL against CpG B alone treated BCP-ALL cell lines KOPN-8 and RS4;11 and CTL against CpG B plus IL-4 plus CD40L treated BCP-ALL cell lines KOPN-8 and RS4;11. These CTL were tested against the same but untreated BCP-ALL cell line. As indicated in figure 10 A, lysis induced by CTL generated with BCP-ALL cells treated with CpG B alone is slightly higher than lysis induced by CTL generated with untreated BCP-ALL cells. CTL generated with BCP-ALL cells treated with CpG B, IL-4 and CD40L, however, were highly more effective against both shown cell lines. Furthermore, the graphs show a direct relationship between effector-target ratio and amount of lysis, which raises, when the effector-target ratio raises. The two lower graphs of figure 10 A show that combination treatment of the stimulator cells with CpG B, IL-4 and CD40L even leads to antigen-specific lysis. Evidently, when RS4;11-specific CTL were incubated with untreated target cells of the third party cell lines Nalm-6 and MHH-CALL-2 no significant lysis occurred, pointing out CTL specificity. Of note, CTL raised against the cell lines Nalm-6 and MHH-CALL-2 acted analogical to the low reactivity of surface molecules of these two cell lines. Even when CTL were raised with Nalm-6 cells that were pretreated with CpG B, IL-4 and CD40L, we could only detect very low specific lysis of untreated
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Nalm-6 target cells. A difference between pretreated and untreated stimulator BCP-ALL cells, however, could still be seen. To translate our cell line results to primary leukemia cells, we repeated the same experimental setting with Xeno BCP-ALL cells, that maintain many features of primary cells. Figure 10B indicates that, although in average less high (figure 11), CTL generated with CpG B, IL-4 and CD40L pretreated Xeno BCP-ALL cells again induced more lysis of untreated target Xeno BCP-ALL cells than CTL generated with untreated Xeno BCP-ALL cells. Furthermore, the detected lysis again was antigen-specific, because third party target cells of another BCP-ALL cell type were left untouched.
Results

Figure 10: Combination treatment with cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B), interleukin- (IL-)4 and cluster of differentiation (CD) 40 Ligand (CD40L) of B cell precursor-acute lymphoblastic leukemia cells (BCP-ALL cells) has a higher stimulatory potential than treatment with CpG B alone in inducing anti-leukemic cytotoxicity. BCP-ALL cells were incubated with CpG B (2.5 µg/ml) alone, or CpG B (2.5 µg/ml) with IL-4 (400 U/ml) and CD40L (1 µg/ml) along with an enhancer for ligands (1 µg/ml) for 48 hours. These cells were added as stimulator cells to freshly isolated peripheral blood mononuclear cells (PBMC) from healthy donors at an effectorto:stimulator ratio of 10:1 to generate anti-leukemic cytotoxic T lymphocytes (CTL). Restimulation with BCP-ALL cells was performed weekly and recombinant human Interleukin 2 (30 U/ml) was added at day 4 after each stimulation cycle. At day 6 of each stimulation cycle 51Cromium-release assay was performed with untreated BCP-ALL cells as target cells. As control served CTL generated with untreated BCP-ALL cells. Line graphs show percentage of specific lysis of untreated target cells as representative results of at least three independent experiments. Percentage of specific lysis was calculated as % specific release = (experimental release - spontaneous release) / (maximum release - spontaneous release) x 100. Error bars indicate standard error of means of experimental triplicates. To confirm the specificity of the anti-leukemic CTL BCP-ALL cell lines Nalm-6 and MHH-CALL 2 were used as third party control. 9A: As BCP-ALL cells served the BCP-ALL cell lines RS4;11 and KOPN-8, in 9B we used Xeno BCP-ALL cells expanded in NOD-SCID mice.
3.5.2 Cytotoxic potential of specific weekly stimulated anti-BCP-ALL CTL remains stable over time and shows a significantly higher mean specific lysis

Additionally to the finding that pretreatment of stimulator BCP-ALL cells enhances their capacity to raise efficient anti-leukemic CTL, we could detect an alteration of cytotoxicity after repeated stimulation cycles. As shown in figure 11, cytotoxicity of CTL raised with pretreated BCP-ALL cells stayed the same or even increased over the weekly restimulations, whereas CTL raised with untreated BCP-ALL cells lost their cytotoxic potential with every restimulation. This effect occurred with the tested cell lines as well as with Xeno BCP-ALL cells. In the experiments with BCP-ALL cell lines, we could show a significant difference between CTL raised with treated stimulator BCP-ALL cells to those raised with untreated. The same experiments with Xeno BCP-ALL cells show the same tendency, but are not yet significant. Here more experiments are needed.

In some CTL generated with Xeno BCP-ALL cells we found that it took repeated stimulation before pretreated BCP-ALL cells turned out to raise more efficient CTL than untreated BCP-ALL cells. To detect a possible relation between upregulation of surface molecules after treatment with CpG B, IL-4 and CD40L and the subsequent stimulatory capacity to induce measurable anti-leukemic cytotoxicity in CTL, we correlated CD54 upregulation and specific lysis of CTL raised with exactly those cells. But for this molecule we could not find a correlation.
Figure 11: Cytolytic potential of cytotoxic T Lymphocytes (CTL) remains stable or rises with weekly stimulation. As described in figure 10, CTL were generated by weekly stimulation with either pretreated or untreated B cell precursor acute lymphoblastic leukemia cells (BCP-ALL cells). Analysis of cytotoxicity was done by 51Cromium-release with untreated BCP-ALL cells as target cells. Bar graphs show average values of specific lysis of untreated BCP-ALL cells after one to four stimulation cycles, S1-S3 with at least three, S4 with two independent experiments. Grey bars indicate untreated stimulator BCP-ALL cells, black bars indicate cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) + interleukin- (IL-) 4 + CD40 ligand treated stimulators. Error bars indicate standard error of means. * indicates p<0.05, ** indicates p<0.01.

3.5.3 Xeno BCP-ALL cells are able to induce cytotoxicity in autologous PBMC after pretreatment with CpG B, IL-4 and CD40L

Cellular therapy strategies always have to deal with the problem to minimize graft-versus-host-disease on the one hand and to keep graft-versus-leukemia reactions strong enough. One back door out of this dilemma could be, not to inject allogeneic cells as cellular therapy, but to work with autologous or HLA-matched cells. For this approach we worked with PBMC derived from pediatric BCP-ALL patients in remission to raise CTL and used the leukemia cells of the identical patient as stimulators and target cells. The leukemia cells were taken at the time point of diagnosis, expanded in the NOD/SCID mice model described above, cryopreserved and thawed directly before use. PBMC were isolated from peripheral blood obtained 6 months after the last anti-leukemic chemotherapy. We used 51Chromium-Release assays to confirm the induction of cytotoxicity in PBMC against their autologous Xeno BCP-ALL cells. Experiments of two individual patients showed that pretreatment of Xeno BCP-ALL cells with
CpG B, IL-4 and CD40L leads to higher specific lysis of untreated Xeno BCP-ALL cells by autologous CTL than by CTL raised with untreated autologous Xeno-BCP-ALL cells. As expected in experiments with autologous cells specific lysis was lower than in experiments with allogeneic cells, but pretreatment of stimulator cells still augmented specific lysis of the untreated target cells by 3-4 fold (figure 12).

**Figure 12:** Pretreated Xeno B cell precursor-acute lymphoblastic leukemia cells (Xeno BCP-ALL cells) are able to induce anti-leukemic cytotoxicity in autologous peripheral blood mononuclear cells (PBMC). PBMC were derived from pediatric BCP-ALL patients in remission at least six month after chemotherapy, those were thawed, after the matched Xeno BCP-ALL cells were stimulated for 48 hours with cytosine-phosphate-guanosine oligodeoxynucleotide B (CpG B) (2.5 µg/ml), interleukin-(IL-)4 (400 U/ml) and cluster of differentiation (CD) 40 ligand (CD40L) (1 µg/ml) along with an enhancer for ligands (1 µg/ml). To generate anti-leukemic cytotoxic T lymphocytes (CTL) restimulation was performed in the same way as described for generation of allogenic anti-leukemic CTL. Again at day 6 of each stimulation cycle 51Cr-release assay was performed with untreated autologous Xeno BCP-ALL cells as target cells. As control served CTL generated with untreated autologous Xeno BCP-ALL cells. Line graphs show percentage of specific lysis of untreated target cells as results of two independent experiments. Percentage of specific lysis was calculated as described in figure 10.
4 Discussion and outlook

The development of an approach that enables the immune system to act against tumor cells is a promising aim in immunotherapy. In this study we aimed at activating B cell leukemia cells in a way that their enhanced immunogenicity induces potent specific anti-tumor cytotoxic T cells. Our results show that treatment of BCP-ALL cells with the TLR9 agonist CpG B in combination with IL-2 family cytokines and CD40L leads to an enhancement of their immunogenicity. Especially CpG B in combination with the T helper cell signals IL-4 and CD40L induce immunologically relevant changes. These changes are represented by increased surface molecule expression, secretion of proinflammatory cytokines, by induction of T cell proliferation and specific cytotoxicity.

It is known that leukemia cells have a low immunogenicity compared to non-malignant cells. This is represented by low expression of surface molecules and the missing ability to induce T cell proliferation in vitro [4], [2]. Leukemia cells even induce tolerance in T cells through the combination of antigen presentation in MHC molecules without a costimulatory signal through CD80/CD28 [4]. But, as other authors showed for B-CLL, lymphoma and leukemia cells, it is possible to overcome this low immunogenicity by stimulation with Toll-like receptor-9-agonists (TLR9) such as CpG B [23], [22]. CpG B is a "danger signal" for the immune system, as it mimics bacterial DNA fragments that bind to TLR9 and induce upregulation of costimulatory and antigen presenting molecules, secretion of proinflammatory cytokines and clonal expansion in normal and malignant B cells [29], [23], [20], [12]. According to other authors, it is even possible to impede the development of newly transplanted leukemia in mice, via intravenous injection of CpG B [16]. In addition, a long-time protection from leukemia has been reported in newly transplanted mice after they had been pretreated with CpG administration before [43]. CpG ODN have been tested as adjuvants to chemotherapy or radiation in clinical experiments on numerous tumors [24], [28], [50]), but the results in human beings are not yet satisfying. Additionally, there are arguments against in vivo application of immune stimulatory agents like CpG ODN in human beings. In-vivo-administered doses of these stimulants cannot be sufficiently controlled, because of plasma protein binding [21] and liver metabolism. Much higher concentrations of
potent stimulants can be used in vitro. Moreover, the success of the approach, for example generation of CTL, can be controlled easily in vitro. In contrast, in vivo there are many parameters eventually skewing the results. Adoptive transfer of "trained" and expanded T cells ex-vivo may be a promising alternative. Our study confirms the stimulatory effect of CpG B, detected by upregulation of costimulatory and antigen presenting surface molecules on BPC-ALL cells. Moreover, we could increase the effect of CpG alone by using IL-4, an IL-2 family cytokine and additional CD40L. The reason to take IL-4 is based on its membership in the IL-2 cytokine family and the availability of IL-2 cytokine family receptors on normal B cells and malignant B cells. In addition, IL-2 receptors are positively regulated on B-CLL cells by CpG B administration. IL-4, as T helper cell cytokine, increases expression of MHC class II molecules on B cells, promotes B and T cell growth and activation, regulates immunoglobulin (Ig) class switch and antibody secretion. In Ig class switch and Ig production IL-4 cooperates with IL-21, another part of the IL-2 cytokine family. On this background, we also tested the combination of CpG B with IL-4 and IL-21. But in our further investigation on CTL generation, we abandoned this combination, due to clearly decreased viability in some BCP-ALL subsets after treatment with IL-21, making them inapplicable as CTL stimulator cells. Further, the combination of CpG B with IL-4 and CD40L turned out to be more effective.

CD40L, the second additional immunostimulatory agent we used, is also called CD154. It is expressed on activated T helper cells and binds to CD40. Moreover, it is known to be a costimulatory molecule and an important regulator of cellular and humoral immunity. It is constitutively expressed on B cells, dendritic cells, monocytes and macrophages. The engagement of CD40 and CD40L leads to activation and maturation of B and T cells. In B cells the engagement results in increased antigen presentation, clonal expansion, cytokine production and antibody secretion and T cells differentiate to helper and cytotoxic T cells. Thus, CTL generation is supported by the help of maturated antigen presenting cells, which polarize T cell differentiation to T helper 1 (Th1) cells, thereby favoring the generation of cell-mediated immunity. Additionally, ligation of CD40 and CD40L can act as alternative "second signal" for T cells and is even able to overcome T cell tolerance. Importantly, while CD4+ T cells express IL-4 and CD40L in a physiologic environment, expression is significantly impaired in bone marrow of ALL mouse models. This could be partially restored with CpG B. In the last years, clinical and preclinical approaches take advantage of the CD40-CD40L pathway, such as using agonistic anti-CD40 antibodies or recombinant human CD40L in cancer patients or in tumor mouse models after adoptive
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T cell therapy [30]. But, although in these phase I studies partial remissions have been observed in some cases and adverse effects only occurred in low to moderate rates, the aim for new research should be further enhancement of the anti-tumor effect. Especially the combination of the agents affecting the CD40 pathway such as TLR agonists appears to be a promising approach [1].

In our experiments we saw an effect of this combination in surface molecule upregulation on BCP-ALL cells as well as an increased stimulatory capacity. The surface molecules that were significantly upregulated were CD40 and CD54. Less upregulation occurred in CD86 and in the antigen presenting molecules MHC class I and II. The combination of CpG B with IL-4 and CD40L showed a significantly higher effect than single treatment. Other studies could show upregulation of the same surface molecules on malignant B cells with CpG alone [22]. CD54, an intercellular adhesion molecule, is not only important for adhesion and diapedesis, but also for the crosstalk between T and B cells. It was shown that interaction between CD54 and LFA-1 can serve as "second signal" for CD8+ T cells that usually is transmitted by CD80/CD86 to CD28 on the T cell [13]. Upregulation of CD54 on antigen-presenting cells (APC) allows multiple adhesion pathways to amplify the immune response. Thus, CD54 can advance generation of CTL after binding of the T cell receptor to MCH class I. Summing up, we found possibilities to support T cell maturation, utilizing CD40-CD40L ligation and CD54-LFA-1 ligation as costimulatory "second signal".

In our study we detected a significantly stronger effect of the combination of IL-4 and CD40L with CpG B than of the single agents. Searching for the mechanism of the synergy, we performed real time PCR for TLR9 receptor mRNA. We found that the combination of IL-4 and CD40L positively regulates TLR9 receptor transcription of BCP-ALL cells, an effect that was enhanced by CpG B. CpG B alone already increased the amount of TLR9 receptor mRNA by itself, but in a smaller amount and not in all cell lines tested. Beyond this it is already described that CpG B also upregulates the essential receptors CD40 [22] and IL-2 cytokine receptors [11]. So we can assume that the synergistic effect is due to the bilateral upregulation of receptors providing more binding sites and thus leading to a stronger stimulating effect.

Beyond this, other authors describe the importance of cytokines for T cell stimulation [26], [9], [8]. Especially, IL-1 beta, IL-12 and Type I INF are proven to be the required "third signal" to develop an effective and long-lasting response of CD8+ T cells. Studies could show that only after priming in the presence of IL-12 or INF-α, CTL developed into memory cells after adoptive transfer in mice, which were able to re-expand and react to new antigen [10], [44]. Therefore, the "third signal" has to
occur after stimulation of the T cell with antigen and CD28 ligation, with IL-12 being required particularly for Th1 cell commitment, a major contribution to effective immunotherapy [26] [8]. In our experimental setting, this precondition may be achieved by IL-12 secretion of the treated BCP-ALL cells. Apart from significant IL-12 secretion by stimulated BCP-ALL cells, we found increased amounts of tumor necrosis factor α (TNF-α), a cytokine usually produced by Th1 cells that are primary responsible for activating CTL development [26]. Thus, the increased secretion of IL-12 and TNF-α by stimulated BCP-ALL cells in our study, may support an efficient anti-leukemic immune response. Consequently we can assume, stimulation of BCP-ALL cells with CpG B, IL-4 and CD40L promotes pro-inflammatory reactions, creating a milieu that promotes the priming and maturation of CD4+ and CD8+ T cells. Hence, the combination of reagents is a step towards the aim to strengthen the immune defense against leukemia, opening ways for new therapeutic approaches.

One question we tried to answer was, if the detected pro-inflammatory reaction can be applied to more physiological assays, like mixed lymphocyte reactions (MLR). In MLR, enhancement of immunogenicity can be detected by induction of proliferation in T cells. We saw a high potential of stimulated Xeno BCP-ALL cells to induce proliferation in T cells. Here again, we could prove the superiority of combining CpG B with IL-4 and CD40L over single treatment, even though we could already observe detectable results with single treatment alone. This is in line with recent data, describing a higher T cell proliferation after CD40L pretreatment of ALL cells compared to non-pretreated ALL cells [4]. In our study, proliferation occurred in both T cell subsets, CD4+ and CD8+ cells, with more distinct proliferation in CD4+ T cells. Of note, this difference in proliferation rate cannot be a direct effect of IL-4, as pretreated BCP-ALL cells were separately stimulated and washed before being added to the MLR. In our MLR experiments, we did not differentiate between CD4+ Th1 and Th2 cells that act in totally different directions. Thus, knowing that pretreatment of the BCP-ALL cells induces secretion of IL-12 and TNF-α, it is likely that the counted CD4+ T cells are Th1 cells, which in turn favor the development of CD8+ cytotoxic T cells. An advantage of proliferation of both T cell subsets could be the cooperation between CD8+ T cells and CD4+ T cells, promising a stronger long-lasting response. Furthermore, CD4+ T cells have the advantage of being able to integrate innate and adaptive immunity and to facilitate cross-presentation of primarily intracellular protein of tumor cells. Thus, through stimulation of both, CD4+ and CD8+ T cells, it seems likely that the development of specific anti-leukemia cytotoxicity is favored. In our experimental setting we tried to allow physiological interaction of T cells with other immune cells.
by using PBMC instead of isolated T cells. Therefore one can assume that antigen presenting cells may have been involved in CTL generation by taking up dying leukemia cells and by presenting leukemia-cell-associated antigens to T cells, as it already has been shown [43].

Even more important than induction of proliferation seems to be the ability of immunogenized BCP-ALL cells to induce cytotoxicity in T cells. In our study, we could induce specific cytotoxicity against untreated BCP-ALL cells, in CTL that were generated with pretreated BCP-ALL cells of the same leukemia. The specific lysis of CTL generated with BCP-ALL cells pretreated with the triple combination of CpG B plus IL-4 plus CD40L was significantly higher than of CTL generated with BCP-ALL cells pretreated with CpG B alone. These results suggest that the combination of CpG B with other T helper cell signals markedly augmented the capacity of such treated BCP-ALL cells to stimulate efficient CTL. The aim to induce anti-leukemic cytotoxicity has already been addressed by many other authors in diverse approaches. For example, specific CTL were generated against ETV6-AML1 protein and were capable of lysing cells expressing this protein endogenously or after transfection [48]. Others were able to induce proliferation of autologous anti-pre B leukemia-specific CTL by priming PBMC with ALL cells pretreated with soluble CD40L and expanding them with the help of repeated administration of IL-2 and ALL stimulator cells [5]. But this group did only succeed in generating CTL from bone marrow, but not from peripheral blood. Others treated BCP-ALL-bearing NOD/SCID mice with CpG in vivo, which resulted in increased systemic leukemic cell death [16]. But NK cell depletion significantly reduced in vivo anti-leukemic activity of CpG, revealing the innate immune system as one decisive source of cytotoxicity. However, in another in-vivo-B cell leukemia mouse model, in which leukemia as well as T cells were of murine origin, the same group identified T cells to be the crucial cells to develop an effective and durable protection against leukemia and proved that an autologous approach is feasible [43].

To pave the way for a potential autologous clinical approach, we also established experiments to test induction of cytotoxicity in an autologous human in-vitro system, by using PBMC and BCP-ALL cells from the same patient. Even in the less stimulatory environment of autologous cells, the pretreatment of BCP-ALL cells revealed to be more efficient in terms of CTL stimulation. As expected, autologous generated CTL induced a lower, but still specific and durable lysis of untreated target BCP-ALL cells. According to our data, the achieved cytotoxicity was specific for the leukemia used to generate the CTL population, tested with untreated cells of other BCP-ALL cell lines as third party. For generation of CTL we did not use any additional stimulator
but weekly stimulation with low-dose IL-2, because development of more unspecific CTL after extended stimulation with higher doses or more frequent IL-2 treatment was described [5]. Thus, we can attribute most of the effect on proliferation and CTL differentiation to the pretreated BCP-ALL cells with enhanced costimulatory and antigen presenting capacity as well as cytokine secretion. Interestingly, others succeeded in generating efficient AML-specific CTL by using allogeneic PBMC with the help of numerous cytokines such as IL-12, IL-7 and IL-15 [14]. These results raise hope for extending our potentially therapeutic approach on other B or T cell malignancies, even though prestimulation would have to be adjusted to the particular malignant cell type. This is supported by a study that proves the feasibility of generating leukemia specific CTL lines for patients with CML, AML and ALL. The leukemia cells were pre-treated with granulocyte-macrophage colony-stimulating factor, stem cell factor, IL-4 and TNF-α [32]. But especially in generating CTL lines against ALL cells success rates were comparatively low suggesting that pretreatment of stimulator ALL cells clearly needs to be optimized. The immunogenic cocktail identified in the study we present here, appears to be more promising for effective anti-BCP-ALL CTL, but further studies are needed to improve anti-T-ALL CTL generation.

The concept of application of our results in future studies in our laboratory is to transfer human leukemic B cells to immunodeficient mice. After establishment of leukemia in vivo, we meanwhile intend to purify and store PBMC either of an HLA-matched or partially HLA-matched unrelated donor, or PBMC of the same patient. As for this study, leukemia cells will be stimulated with CpG B, IL-4 and CD40L. Subsequently, CTL will be generated from allogeneic or autologous PBMC with the help of pretreated leukemia cells. After successful generation of leukemia-specific CTL, they will be purified and adoptively transferred back to the mice. This concept aims at diminishing graft versus host disease by using HLA-matched or autologous T cells and still maintaining specific anti-leukemic cytotoxicity of CTL. Surely, in a clinical setting, this kind of therapy may only play an additional part in connection with chemotherapy, because of established protocols and because of its presumably lower strength. But because of its specificity, this therapy could prevent relapses that are difficult to treat by controlling and diminishing residual tumor cells. In relapsed acute myeloid leukemia after reduction of tumor cell load, immunotherapy is already tested as adoptive T cell transplantation or active immunization for example with loaded dendritic cells. Here, interesting positive effects, like reduction of minimal residual disease and tumor reduction were seen, though still strong adverse effects occurred [25].

First attempts have been made to integrate treatment with leukemia-specific CTL in
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After allogeneic bone marrow transplantation, patients received an infusion with in-vitro-generated leukemia-specific CTL. Here donor T cells were stimulated with HLA-identical leukemic antigen presenting cells, including malignant B cells, after stimulatory pretreatment in an in vitro culture system [32]. First results showed feasibility of this approach, but further studies are needed to confirm these results and to optimize stimulator leukemia cell treatment as well as anti-leukemic cytotoxicity. Another aspect is the state of differentiation of the stimulated and transferred T cells. It has been published that T cells in a higher state of differentiation that were adoptively transferred in an in-vivo melanoma-mouse model caused a less effective tumor eradication than lower differentiated T cells [18]. In addition the authors show a system of stimulation with the help of APC, an approach that may be considered in our future projects.

We conclude, that pretreatment of BCP-ALL cells with a cocktail containing CpG B, CD40L and IL-4 profoundly enhances the immunogenicity of these leukemic cells, which can be efficiently used to generate both allogeneic and autologous CTL, with specific cytotoxicity towards untouched BCP-ALL cells. Further investigations in a humanized mouse model need to confirm the efficacy of an adoptive CTL transfer approach as proposed by our work. A future in-vivo-project may contribute to answer the following questions: Do the transferred CTL survive in vivo? Are they capable of lysing leukemia cells in vivo? How many CTL do we need to induce an effective response? How can enough CTL be generated and when is the best time point of transfer? Does this treatment result in graft versus host disease? Is overall survival affected?

Based on our results presented a step towards a novel immunotherapeutic approach to the management of BCP-ALL may be taken.
5 Summary

Leukemias represent the most common childhood malignancies, most of them B cell precursor acute lymphoblastic leukemias (BCP-ALL). The poor prognosis of the children in the high-risk group and a survival rate of only 60% in adults call for further investigation on therapeutic options. One interesting option may be development of new immunotherapeutic approaches. Their low immunogenicity enables BCP-ALL cells to escape anti-tumor mechanisms by low expression of surface and costimulatory molecules. Still, expression of Toll-like receptors (TLR) and interleukin- (IL-) 2-family receptors offers the possibility to stimulate BCP-ALL cells through cytokines and TLR agonists. It was demonstrated that malignant B cells develop a more pronounced immunogenic phenotype by upregulation of major histocompatibility complex (MHC) and costimulatory molecules when stimulated with CpG oligodeoxynucleotides, ligands of TLR9, the most abundant TLRs on B cells. The aim of this study was to investigate the effect of CpG B, the IL-2 family cytokine IL-4 and cluster of differentiation (CD) 40 ligand (CD40L) on the immunogenicity of BCP-ALL cells. The rationale to use IL-4 and CD40L was based on the finding that CpG B positively regulates IL-2 family receptors and CD40, receptor of CD40L and the known immunogenic effects of these stimulants on normal B cells. For this purpose, we treated either various BCP-ALL cell lines or patient-derived BCP-ALL cells previously expanded in NOD/SCID mice (Xeno BCP-ALL cells) in an in-vitro-culture model with various combinations of CpG B, IL-4 and CD40L. We analyzed surface molecule expression and the stimulating effect of the treated BCP-ALL cells on T cells. Methods we used were flow cytometry, CFSE proliferation assays, cytometric bead array, polymerase chain reaction (PCR) and Chromium release cytotoxicity assays. We could show that the combination of CpG B, IL-4 and CD40L by far exceeds the effect of CpG alone, demonstrated by upregulation of surface molecules, such as CD40, CD54 and MHC class I and II, supporting communication of these malignant cells with immune cells. Furthermore, Xeno BCP-ALL cells stimulated with the combination of CpG B, CD40L and IL-4 were able to induce significantly higher proliferation in human allogeneic freshly isolated peripheral blood mononuclear cells (PBMC) than only CpG B stimulated cells. The
results obtained by CFSE-staining could be confirmed with \(^3\)H-Thymidine incorporation analysis. Using cytometric bead array we could show that stimulated BCP-ALL cell lines secrete TNF-\(\alpha\) and IL-12, hence creating an immunogenic milieu that promotes the maturation and priming of T cells. To learn more about the mechanisms of the synergistic effect of CpG B with IL-4 and CD40L, PCR of TLR9 mRNA (messenger ribonucleic acid) expression was performed. We observed an increase of TLR9 mRNA after stimulation with CD40L and IL-4, which may explain the additional stimulatory effect of CpG. On the other hand, upregulation of CD40 by CpG increased responsiveness to soluble CD40L. The triple combination furthermore proved to be significantly more effective in inducing anti-leukemic cytotoxic T lymphocytes (CTL). Importantly, such CTL expressed antileukemic cytotoxicity not only against treated but also against untreated BCP-ALL cells. We could show that this effect is specific by using third party BCP-ALL cells. With the long-term therapeutic aim to enable transfer of antileukemic CTL to patients, we tested our findings in an autologous setting with BCP-ALL cells expanded in NOD/SICD mice and PBMC derived from the same patient at least 6 months after chemotherapy. We observed the same tendency, even though at a lower level.

Taken together, our results demonstrate that the triple combination of CpG B with IL-4 and CD40L is more efficient than CpG alone in profoundly increasing immunogenicity of BCP-ALL cells by mutual upregulation of their respective binding partners. Furthermore, we could raise effective allogeneic and autologous anti-B cell leukemia CTL against untouched BCP-ALL cells. We conclude that the immunogenic cocktail we tested here, may be an efficient tool to overcome poor recognition of BCP-ALL cells by immune cells in vitro and that these malignant cells with regained immunogenicity may be used to "train" immune cells in vitro for improvement of anti-leukemic immunity. Further investigations are needed to confirm our results and to study possible advantages of a more complex priming and maturation system of CTL. An extension of our approach may be the additional use of antigen-loaded dendritic cells. The next step will be to test the feasibility and effectiveness of adoptive anti-leukemic T cell transfer in a humanized mouse model, paving the way for a novel cellular therapy in B cell leukemias.
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