A human monoclonal antibody drug and target discovery platform for B-cell chronic lymphocytic leukemia based on allogeneic hematopoietic stem cell transplantation and phage display

Category: Transplantation

Running head: Mining antibody repertoires after transplantation

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Abstract

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is the only potentially curative treatment available for patients with B-cell chronic lymphocytic leukemia (B-CLL). Here we show that post-alloHSCT antibody repertoires can be mined for the discovery of fully human monoclonal antibodies (mAbs) to B-CLL cell surface antigens. Sera collected from B-CLL patients at defined time points after alloHSCT revealed selective binding to primary B-CLL cells. Pre-alloHSCT sera, donor sera, and control sera were negative. In order to identify post-alloHSCT serum antibodies and subsequently the B-CLL cell surface antigens they recognize, we generated a human Fab library from post-alloHSCT peripheral blood mononuclear cells (PBMC) and selected it on primary B-CLL cells by phage display. A panel of Fab with B-CLL cell surface reactivity was strongly enriched. The selection was dominated by highly homologous Fab predicted to bind the same antigen. One Fab was converted to IgG1 and analyzed for reactivity with PBMC from B-CLL patients and healthy volunteers. Cell surface antigen expression was found to be restricted to primary B cells and up-regulated in primary B-CLL cells. Mining post-alloHSCT antibody repertoires offers a novel route to discover fully human mAbs and identify antigens of potential therapeutic relevance to B-CLL and possibly other cancers. Trials described herein have been registered with www.clinicaltrials.gov under identifiers NCT00055744 and NCT00003838.
Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is a biologically and clinically heterogeneous hematologic malignancy characterized by a gradual accumulation of proliferating, resting, and dying CD5+ CD19+ CD23+ monoclonal B cells. Monoclonal antibodies (mAbs) alone or in combination with chemotherapy, hold substantial promise for first-line and second-line treatment of B-CLL. However, most preclinically and clinically investigated mAbs for the therapy of B-CLL target cell surface antigens are also expressed by healthy B cells and other blood cells of lymphoid and myeloid lineage. By contrast, mAbs to cell surface antigens that are unique to or at least over-expressed on B-CLL cells may be less toxic and more active by allowing selective intervention with powerful antibody-drug conjugates, immunotoxins, and radioimmunoconjugates. A few differentially expressed B-CLL cell surface antigens that may be suitable for selective mAb therapy have been discovered through gene expression profiling. A more direct antigen discovery strategy, termed SEREX, utilizes serum antibodies from cancer patients for the screening of cDNA expression libraries. However, antigens that were identified by SEREX in a variety of cancers, including B-CLL, are predominantly intracellular proteins that do not allow mAb targeting. On the other hand, SEREX has become a valuable tool for the discovery of T-cell antigens because serum antibodies to intracellular proteins can induce CD8+ T-cell responses to peptide epitopes within the antigen by cross-presentation mediated through Fcγ receptors on dendritic cells.

SEREX has also been applied to the discovery of antigens that mediate graft-versus-leukemia (GVL) activity following allogeneic hematopoietic stem cell transplantation (alloHSCT). Currently, alloHSCT is the only potentially curative treatment available for patients with B-CLL. Strong GVL activity is evident in B-CLL following alloHSCT from human leukocyte antigen (HLA)-matched related and unrelated donors. GVL and its counterpart graft-versus-host-disease (GVHD) are believed to be mediated primarily by alloreactive donor T cells that recognize minor histocompatibility antigens, i.e. HLA-displayed peptides derived from polymorphic proteins that are different in recipient and donor. In addition, GVL activity may be mediated by HLA-displayed peptides derived from antigens that are selectively expressed or over-expressed in leukemia cells.
Shifting the focus to another component of the adaptive immune system, there is growing interest in investigating whether alloHSCT-induced antibodies derived from donor B cells may also have a role in GVL activity, either indirectly through cross-presentation of antigens for induction of CD8+ T-cell responses or directly through tumor cell surface targeting.\textsuperscript{17} Using SEREX, serum antibodies from alloHSCT patients who had received donor lymphocyte infusion (DLI) led to the identification of potential GVL antigens in chronic myelogenous leukemia\textsuperscript{18-21} and multiple myeloma.\textsuperscript{22,23} Even for alloHSCT patients who had not received DLI, SEREX identified candidate GVL antigens in mantle cell lymphoma\textsuperscript{24} and adult T-cell leukemia.\textsuperscript{25} Alloreactive antibodies directed against H-Y antigens encoded on the Y chromosome, including minor histocompatibility antigen DBY, were discovered in male recipients with female donors.\textsuperscript{26,27} While the vast majority of candidate GVL antigens discovered by SEREX were intracellular proteins, several cell surface proteins that may mediate direct cytotoxicity of post-alloHSCT serum antibodies have also been identified.\textsuperscript{23,25,28} Collectively, these studies suggest that candidate GVL antigens in B-CLL may be discovered through post-alloHSCT serum antibodies, including cell surface antigens suitable for selective mAb therapy.

Here we investigate the hypothesis that alloHSCT induces a serum antibody response to B-CLL cell surface antigens that can be harnessed for human mAb drug and target discovery through the generation and selection of post-alloHSCT antibody libraries. In clear contrast to SEREX, our approach was designed to (i) confine target discovery to cell surface antigens and (ii) concomitantly yield fully human mAbs of potential therapeutic utility. B-CLL in the context of alloHSCT may be particularly suited for this approach due to the typically slow disappearance of B-CLL cells.\textsuperscript{12,29} The continued presence of B-CLL antigens in the first several months after alloHSCT may support the formation of secondary antibody repertoires characterized by somatic hypermutation, class switch recombination, and receptor editing of immunoglobulin (Ig) genes in the reconstituting B-cell compartment.\textsuperscript{30,31}
Materials and methods

Clinical samples
Untreated B-CLL patients (n=13) as well as alloHSCT-treated B-CLL patients (n=2) along with their HLA-matched sibling donors were enrolled in institutional review board approved protocols at the Clinical Center, National Institutes of Health (NIH) (Bethesda, MD), and all patients gave informed consent in accordance with the Declaration of Helsinki. Plasma from B-CLL patients and donors was prepared from blood and stored at -80°C. Peripheral blood mononuclear cells (PBMC) were prepared from blood using Lymphocyte Separation Medium (MP Biomedicals, Solon, OH) and cryopreserved until use. PBMC from healthy volunteers (n=11) were prepared from freshly drawn blood obtained from the Department of Transfusion Medicine, Clinical Center, NIH. CD19+ and CD19- subpopulations were purified from human PBMC by magnetic activated cell sorting (MACS) using CD19 MicroBeads (Miltenyi Biotec, Auburn, CA).

Cell lines
Epstein-Barr virus (EBV) transformed B lymphoblastoid cell lines (EBV-LCL) were generated from PBMC of a healthy volunteers (0745) and B-CLL patients (18-7-3 and 18-1-12) as described.\textsuperscript{32} Using PCR amplification of Ig heavy chain VDJ gene fragments from genomic DNA\textsuperscript{33}, EBV-LCL 0745 was found to be polyclonal as indicated by multiple bands. The same analysis revealed that EBV-LCL 18-7-3 and 18-1-12 were monoclonal albeit different in HCDR3 lengths than the corresponding B-CLL cells, suggesting EBV transformation of normal B cells present in these B-CLL patients. EBV-LCL 583 and 1363 were generated from PBMC of melanoma patients and kindly provided by Dr. Suzanne L. Topalian (Surgery Branch, National Cancer Institute, NIH). B-CLL cell line EHEB was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).\textsuperscript{34} B-CLL cell line 232-B4 was kindly provided by Dr. Anders Rosén (Linköping University, Linköping, Sweden).\textsuperscript{35} Human Burkitt’s lymphoma B-cell lines Daudi, Raji, and Ramos and human mantle cell lymphoma B-cell line JeKo-1 were obtained from American Type Culture Collection (Manassas, VA).

Control mAbs
Rituximab (Rituxan®; Genentech, South San Francisco, CA, and Biogen Idec, Cambridge, MA), and alemtuzumab (Campath®; Genzyme, Cambridge, MA) were obtained from the pharmacy of the Clinical Center, NIH. The generation, expression, and purification of human anti-tetanus toxoid mAb TT11 IgG1 was described previously.36

**Flow cytometry**

*Post-alloHSCT serum antibody detection.* Multiparameter flow cytometry was performed in an LSR II instrument (BD Biosciences, Immunocytometry Systems, San Jose, CA). All incubation steps were on ice for 1 h. Approximately 5 x 10⁵ cells PBMC prepared from an untreated B-CLL patient (pilot experiment) or from the two alloHSCT-treated B-CLL patients prior to induction chemotherapy (Table 1) were first blocked with 4% (v/v) normal goat serum in PBS (used for all subsequent dilutions and washes) followed by 100 μg/mL unconjugated goat Fab anti-human IgG polyclonal antibodies (pAbs; Jackson ImmunoResearch Laboratories, West Grove, PA). After two washes, the cells were incubated with 1 μg/mL rituximab or alemtuzumab (pilot experiment) or with a 1:2 dilution of plasma from alloHSCT-treated B-CLL patients and donors or, as negative control, pooled human AB serum (Invitrogen, Carlsbad, CA). Following two washes, the cells were incubated with 20 nM goat F(ab’)₂ anti-human IgG pAbs conjugated to Qdot 655 (Quantum Dot Corporation, Hayward, CA). The cells were co-stained with CD3-FITC/CD19-PE Simulstest reagent (BD Biosciences) for gating T cells and B cells, and propidium iodide for excluding dead cells from the analysis. After two more washes, a total of 20,000 gated events were collected for each sample in a list mode file, and data analysis was performed using FACS Convert and CellQuest software (BD Biosciences).

*JML-1 IgG1 binding.* Approximately 5 x 10⁵ cells (from a cell line, from PBMC of B-CLL patients, or from MACS-purified CD19+ PBMC subpopulations of healthy volunteers) were first incubated with pooled human AB serum for 20 min to block Fcγ receptors. All subsequent incubations were on ice for 1 h. The cells were incubated with different concentrations (0.1-10 μg/mL) biotinylated JML-1 or TT11 IgG1 in 2% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) in PBS (used for all subsequent dilutions and washes), washed twice and incubated with 2 μg/mL PE-coupled streptavidin (BD Biosciences). After two more washes, propidium iodide was added to exclude dead cells from the analysis, and flow cytometry was performed using a FACSCalibur instrument (BD Biosciences). The data was analyzed with CellQuest
software. In case of B-CLL PBMC, the cells were co-stained with CD19-APC (BD Biosciences) for gating CD19+ and CD19- subpopulations.

**Human Fab library generation**

A human Fab library was generated from cryopreserved post-alloHSCT PBMC from one patient ("patient A") collected six months after transplantation. Total RNA was extracted from 2.5 x 10^7 PBMC using TRI Reagent (Molecular Research Center, Cincinnati, OH) and further purified using the RNeasy Mini Kit from Qiagen (Germantown, MD). Approximately 100 μg total RNA was isolated and validated by agarose gel electrophoresis. First-strand cDNA synthesis from total RNA using an oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen) were carried out according to the manufacturer’s protocol. \( V_\kappa \), \( V_\lambda \), and \( V_H \) encoding sequences were separately amplified from first-strand cDNA by a 35-cycle PCR using the FastStart High Fidelity PCR System from Roche (Indianapolis, IN) and combinations of 12 sense/1 antisense primers for \( V_\kappa \), 20 sense/3 antisense primers for \( V_\lambda \), and 19 sense/6 antisense primers for \( V_H \), for a total of 186 different combinations, encompassing all human germlines. (Note: The antisense primers for \( V_\lambda \) and \( V_H \) align to \( J_\lambda \) and \( J_H \) germlines, respectively, whereas the antisense primer for \( V_\kappa \) aligns to the \( C_\kappa \) encoding sequence). Human \( C_\kappa \)-pelB and \( C_\lambda \)-pelB encoding sequences required for the \( V_\kappa \)-\( C_\kappa \)-\( V_H \) and \( V_\lambda \)-\( C_\lambda \)-\( V_H \) cassette assembly, respectively, were amplified from \( pC_\kappa \) and \( pC_\lambda \). \( V_\kappa \)-\( C_\kappa \)-\( V_H \) and \( V_\lambda \)-\( C_\lambda \)-\( V_H \) cassettes were assembled in one fusion step based on 3-fragment overlap extension PCR, digested with SfiI, and cloned into pC3C as described. Transformation of \( E. coli \) strain XL1-Blue (Stratagene) by electroporation yielded 9.8 x 10^7 and 1.6 x 10^8 independent transformants for the \( \kappa \) and \( \lambda \) phagemid libraries, respectively. Randomly picked independent transformants from each library were analyzed for Fab expression by ELISA and for sequence diversity by DNA fingerprinting using \( Alu \)I as described. Using VCSM13 helper phage (Stratagene), the pooled phagemid library was converted to a phage library as described and stored at 4°C after adding sodium azide to a final concentration of 0.02% (w/v).

**Human Fab library selection**

The human Fab library was selected on cryopreserved PBMC (consisting of >85% B-CLL cells) from an untreated B-CLL patient (“patient α”) that had been maintained in 6-well tissue culture plates for 1-2 days in RPMI 1640 (Invitrogen) supplemented with 5% (v/v) autologous serum.
Five rounds of panning were carried out using established phage display protocols. All incubations were at room temperature unless noted otherwise. In the first round, the freshly re-amplified phage library was pre-selected for functional Fab display through panning on rat anti-hemagglutinin (HA) mAb (Roche) immobilized to three wells of a 96-well ELISA plate (Costar 3690; Corning, Corning, NY) at 500 ng/well. In the second round, 0.5 mL of fresh phage was first mixed with 0.5 mL 5% (v/v) autologous serum in PBS and 0.5 mL 1% (w/v) BSA in PBS. After adding sodium azide to a final concentration of 0.12% (w/v), the phage were incubated for 30 min. Primary B-CLL cells from one 6-well tissue culture plate were harvested, collected through Lymphocyte Separation Medium, resuspended in 1.5 mL 5% (v/v) autologous serum in PBS, counted (4.2 x 10⁷), added to the 1.5-mL phage preparation in a 15-mL polypropylene tube, and incubated for 30 min with gentle agitation every 5 min. After washing three times with 15 mL PBS, the cells were resuspended in 0.6 mL PBS containing 10 mg/mL trypsin, shook at 37ºC and 250 rpm for 30 min, and added to two 2-mL XL1-Blue cultures, resuming the phage display protocol. The third round was identical to the second round, except that 1.2 x 10⁷ primary B-CLL cells were used. In the fourth round, selection for functional Fab display was repeated using two wells with immobilized rat anti-HA mAb at 200 ng/well. The fifth round was identical to the second and third round, except that 5 x 10⁷ primary B-CLL cells were used and four washes with 15 mL PBS were carried out. Phage output-to-input ratios after each round were determined through output and input titering as described. One hundred randomly picked clones from the final output were analyzed for Fab expression by ELISA and for sequence diversity by DNA fingerprinting using AluI as described.

Whole cell ELISA

Phage. Based on input titering, polyclonal phage from the second, third, and fifth round were diluted to approximately 1 x 10¹¹ phage in 75 μL PBS and stored on ice. All subsequent steps were carried out in a V-bottom 96-well tissue culture plate (Costar 3894; Corning) at room temperature using PBS for washing and dilution. Approximately 5 x 10⁵ primary B-CLL cells from patient α were washed twice, resuspended in the 75-μL phage preparations, or in PBS as negative control, and incubated for 1 h on a rocker. Subsequently, the cells were washed twice and incubated with 100 μL of a 1:1,000 dilution of mouse anti-phage mAb conjugated to horse radish peroxidase (HRP; GE Healthcare, Piscataway, NJ) for 1 h. Finally, the cells were washed
twice, resuspended in 50 μL HRP substrate solution\textsuperscript{39}, and incubated for 20 min. Absorbance at 405 nm was determined with an ELISA plate reader.

Crude Fab. Following induction of Fab expression with isopropyl β-D-1-thiogalactopyranoside as described\textsuperscript{39}, bacterial supernatants from four to five selected clones with identical fingerprints were pooled and concentrated tenfold using a 15-mL Amicon Ultra Centrifugal Filter Device with 10-kDa molecular weight cut off (Millipore, Billerica, MA). Whole cell ELISA was carried out using 75 μL concentrated supernatant and HRP-conjugated rat anti-HA mAb as described above.

**Generation, expression, purification, and biotinylation of JML-1 IgG1**

For the conversion of JML-1 Fab to JML-1 IgG1, the V\textsubscript{H} and light chain encoding sequences were PCR amplified using appropriately designed primers and cloned into mammalian expression vector PIGG as described.\textsuperscript{37} Using 293fectin, 300 μg of PIGG-JML-1 plasmid was transiently transfected into 3 x 10\textsuperscript{8} HEK 293F cells and kept in 300 mL FreeStyle serum-free medium in a 500-mL spinner flask on a stirring platform at 75 rpm (CELLSPIN System; Integra, Chur, Switzerland) in a humidified atmosphere containing 8 % CO\textsubscript{2} at 37°C. After four days, the medium was collected after centrifugation, replaced for additional three to four days, and collected again. Pooled supernatants were then processed and IgG1 purified using a 1-mL recombinant Protein A HiTrap column (GE Healthcare) as described.\textsuperscript{37} The quality and quantity of purified IgG1 was determined by SDS-PAGE and A\textsubscript{280} absorbance. Purified JML-1 IgG1 and TT11 IgG1 were biotinylated using the BiotinTag Micro-Biotinylation Kit (Sigma-Aldrich, St. Louis, MO). The number of conjugated biotin molecules per IgG1 molecule was equivalent for JML-1 and TT11 IgG1.
Results

Detection of post-alloHSCT serum antibodies with B-CLL cell surface reactivity

To detect presumably low concentrations of human serum antibodies against B-CLL cells in patients following alloHSCT, we developed a sensitive flow cytometry assay using goat F(ab’)\textsubscript{2} anti-human IgG pAbs conjugated to Qdot 655 nanocrystals (Figure 1). The detection of human serum antibodies specifically binding to B-CLL cell surface antigens is complicated by the fact that B-CLL cells typically express transmembrane IgM, IgD, and Fcγ receptors such as CD32B, albeit at lower cell surface densities than normal B cells.\textsuperscript{40} In order to avoid detection of human Ig that are directly (as IgM or IgD) or indirectly (through binding to CD32B) displayed on the B-CLL cell surface, the assay required dual blocking with normal goat serum and unconjugated goat Fab anti-human IgG pAbs. While normal goat serum blocks Fcγ receptors, unconjugated goat Fab anti-human IgG pAbs were intended to block the binding of conjugated goat F(ab’)\textsubscript{2} anti-human IgG pAbs to surface IgM and IgD epitopes that are shared with IgG (such as epitopes in the light chain and variable domain of the heavy chain). We used monovalent Fab for blocking to avoid artificial recruitment of serum antibodies and bivalent F(ab’)\textsubscript{2} for detection to gain avidity. The assay was established using rituximab (a chimeric mouse/human anti-human CD20 mAb with human IgG1κ constant domains) or alemtuzumab (a humanized anti-human CD52 mAb with human IgG1κ constant domains) and PBMC from an untreated B-CLL patient (Figure 1). These pilot experiments suggested the suitability and sensitivity of the assay for detecting the binding of post-alloHSCT serum antibodies to B-CLL cell surface antigens.

A complete set of frozen plasma samples from time points before, at, and after alloHSCT over a period of at least one year was available for two refractory B-CLL patients who had received a nonmyeloablative alloHSCT from an HLA-matched sibling donor on two different protocols at the Clinical Center, NIH, and who remain in molecular remission six and seven years later, respectively (Table 1). For each of the two patients, PBMC from a time point prior to induction chemotherapy served as source of primary B-CLL cells (CD3- CD19+) and T cells (CD3+ CD19-). Using the established assay, post-alloHSCT serum antibodies against B-CLL surface antigens were detected in both patients. Post-alloHSCT plasma from patient A revealed a substantial but transient occurrence of serum antibodies with B-CLL cell surface reactivity,
peaking at six months after transplantation (Figure 2A). A parallel but weaker T cell surface reactivity was also observed. By contrast, pre- and peri-alloHSCT plasma, plasma from the alloHSCT donor of patient A, and pooled control plasma from healthy volunteers were all negative. A similar pattern of serum antibody reactivity was seen for patient B (Figure 2B) from a different protocol. However, the transient B-CLL cell surface reactivity was confined to later time points, peaked at ten months after transplantation, and was not accompanied by T cell surface reactivity. These patterns of serum antibody reactivity seen for patient A and B were reproducible in independent experiments.

The limited availability of post-alloHSCT plasma did not permit an extensive analysis of the reactivity of post-alloHSCT serum antibodies at these defined time points. However, as summarized in Table 2, some cell surface reactivity with allogeneic B cells and third party B cells was noted. Additional analyses with secondary antibodies specific for human Ig isotypes suggested that both IgG and IgM contributed to the transient B-CLL cell surface reactivity (data not shown). When compared to clinical data, the peak in transient B-CLL cell surface reactivity approximately paralleled the time points at which full donor chimerism was achieved and the disappearance of B-CLL cells by flow cytometry and PCR was noted (data not shown). This suggested that the observed serum antibody response against B-CLL cells is an antigen-dependent phenomenon, likely involving both autologous and allogeneic epitopes of cell surface antigens.

**Generation and selection of a post-alloHSCT human Fab library by phage display**

In order to identify post-alloHSCT serum antibodies and subsequently the cell surface antigens they recognize, we utilized phage display vector pC3C36,37 for the generation of a human Fab library from post-alloHSCT PBMC (Figure 3A). Total RNA was prepared from post-alloHSCT PBMC collected from patient A at the peak of serum antibody response (six months; Figure 2A), and human V\(_k\), V\(_\lambda\), and V\(_H\) encoding sequences were amplified by RT-PCR. To include all human germlines, we used a total of 61 primers in 186 different and separate combinations as described previously for the generation of a naïve human Fab library.36 Due to a depleted and not yet fully recovered B cell repertoire in post-alloHSCT PBMC, we expected a smaller number of successful primer combinations when compared to normal PBMC from a healthy volunteer.
While this was indeed the case, the difference was smaller than expected with a success rate of 71% for post-alloHSCT PBMC compared to 89% for normal PBMC (Table 3). This finding along with a library size of 2.6 x 10^8 independent Fab clones implied a high complexity of our post-alloHSCT human Fab library. Its integrity and diversity was confirmed by ELISA and DNA fingerprinting of unselected Fab clones.

The post-alloHSCT human Fab library was selected by three rounds of panning on PBMC from untreated B-CLL patient α (Figure 3B). Two additional rounds of panning on immobilized rat anti-HA mAb were carried out prior to the first and after the second cell panning round in order to eliminate phage that did not display functional Fab with HA tag. The selection was monitored by phage output-to-input ratios (Figure 4A) and by whole cell phage ELISA (Figure 4B), both of which revealed the enrichment of phage displaying Fab with B-CLL cell surface reactivity. Out of 100 selected Fab clones that were analyzed by ELISA, whole cell ELISA on PBMC from untreated B-CLL patient α, and DNA fingerprinting, 85 revealed Fab expression, B-CLL cell surface reactivity, and readable DNA fingerprints. Among these Fab clones, 73 belonged to one of seven repeated DNA fingerprints and 63 belonged to one of four dominating patterns with eight or more apparently identical Fab clones. Representative Fab clones, designated JML-1, -3, -7, and -13, each from one of the four dominating patterns, were further analyzed by DNA sequencing. The deduced amino acid sequences of the variable domains were compared with respect to germline origin, LCDR3 and HCDR3 sequences, and overall homology (Table 4). Strikingly, all four Fab clones were highly homologous with identical V_κ, J_κ, D_H, and J_H germline origins and identical LCDR3 and HCDR3 sequences. However, all Fab clones differed by at least four amino acids, and JML-1 had a different V_H germline origin than JML-3, -7, and -13 despite their identical HCDR3 sequences. A BLAST search of protein databases (http://blast.ncbi.nlm.nih.gov) revealed that this HCDR3 sequence, GGQTIDI, is unique among rearranged heavy chains. Clearly, the high homology of the four dominating Fab clones, in particular their identical LCDR3 and HCDR3 sequences, imply recognition of the same antigen. JML-3, -7, and -13 revealed approximately 2% overall deviation from their V_κ and V_H germlines. Strong evidence for somatic hypermutation was found for the heavy chain of JML-1 with a deviation of approximately 7% from its V_H germline (Table 4). Based on this observation and the fact that crude Fab preparations from pooled clones with the JML-1 fingerprint
consistently revealed the strongest B-CLL cell surface reactivity by whole cell ELISA on PBMC from untreated B-CLL patient α (data not shown), we subsequently focused on JML-1.

**Generation and characterization of JML-1 IgG1**

Using mammalian cell expression vector PIGG\(^4\), JML-1 was converted from Fab to IgG1, expressed in HEK 293F cells, purified, and biotinylated. The previously described human anti-tetanus toxoid mAb TT11 IgG1\(^3\), which was expressed, purified, and biotinylated in the same way as JML-1 IgG1, served as negative control. Flow cytometry confirmed the B-CLL cell surface reactivity; JML-1 IgG1, but not TT11 IgG1, strongly bound to B-CLL cells from patient α that had been used for library selection. JML-1 IgG1 also recognized B-CLL cells from patient A, the alloHSCT recipient from whom the library had been generated (Figure 5A).

Binding of JML-1 IgG1 to the CD19+ subpopulation of B-CLL PBMC was evident with as little as 0.1 μg/mL, whereas no binding to the CD19- subpopulation of B-CLL PBMC was seen with up to 10 μg/mL (data not shown). We next tested whether JML-1 IgG1 can bind to third party B-CLL cells from untreated patients that were not involved in library selection and generation. Eleven out of twelve B-CLL patients tested revealed JML-1 IgG1 but not TT11 IgG1 reactivity (Figure 5B), suggesting that the antigen recognized by JML-1 is broadly expressed in B-CLL. The level of expression in a given B-CLL patient was found to be highly reproducible in independent experiments, but variable among different B-CLL patients. No correlation with favorable or unfavorable prognostic markers of B-CLL\(^1\) was noted (data not shown). When PBMC from healthy volunteers were separated by MACS into CD19+ and CD19- subpopulations, JML-1 IgG1 reactivity that also varied among different individuals was noted only for the CD19+ subpopulation (Figure 5A and B). However, compared to primary B cells, the mean JML-1 IgG1 reactivity measured for primary B-CLL cells was significantly higher (p=0.023, Mann-Whitney test; Figure 5B). Notably, none of eleven human B-cell lines we analyzed revealed any JML-1 IgG1 reactivity. These included two EBV-transformed B-cell lines from B-CLL patients, three EBV-transformed B-cell lines from healthy volunteers, and the previously described B-CLL cell lines EHEB and 232-B4, Burkitt’s lymphoma B-cell lines Daudi, Raji, and Ramos, and mantle cell lymphoma B-cell line JeKo-1 (Figure 5B).

Collectively, these findings suggested that the antigen recognized by JML-1 is restricted to primary B cells and over-expressed in primary B-CLL cells.
Discussion

We report here the generation and selection of what is to our knowledge the first human post-alloHSCT antibody library. Adding to human immune, naïve, and synthetic antibody repertoires, we demonstrate that the human post-alloHSCT antibody repertoire can be successfully mined by phage display to yield fully human mAbs of potential therapeutic relevance. Specifically, we present a strategy for exploiting post-alloHSCT antibody repertoires for concerted drug and target discovery in B-CLL therapy.

Finding a suitable time point for the generation of antibody libraries from post-alloHSCT PBMC is complicated by the fact that B-cell reconstitution is variable and depending on a number of treatment-related and patient-specific parameters. We postulated that suitable time points could be narrowed down by monitoring the appearance of post-alloHSCT serum antibodies with cell surface reactivity which indicates the emerging ability of the reconstituted B-cell compartment to mount a potent serum antibody response. Although a substantial portion of this cell surface reactivity may be mediated by antibodies that are polyspecific, autoreactive, or alloreactive, and therefore of limited use for broader mAb drug and target discovery, recent studies suggest the emergence of post-alloHSCT serum antibodies that selectively bind to tumor cell surface antigens. As we show here, phage display provides a powerful tool for isolating and defining minor specificities of potentially broader utility from the post-alloHSCT antibody repertoire.

The cell surface reactivity we detected in two B-CLL patients following alloHSCT was highly transient with peaks at six months and ten months, respectively. This finding is an agreement with other studies that observed peaks and waves for particular serum antibodies that emerged from post-alloHSCT antibody repertoires. By contrast, post-DLI antibody repertoires that have been mined for tumor specificity using SEREX, appear to sustain a constant level of serum antibodies over longer periods of time. These differences may be inherent to the divergence of reconstituting (post-alloHSCT) and reconstituted (post-DLI) B-cell compartments and may be shaped by innate and adaptive immune responses.
While the reconstituting antibody repertoire is likely dominated by natural antibodies that are part of the innate immune system and provide a first line of defense characterized by polyspecificity and low affinity, JML-1 and its homologues appear to be products of an adaptive immune response driven by the presence of antigen. In fact, the phage display approach we used for the selection of the JML-1 clonotype is based on monovalent Fab and thereby geared for the selection of high affinity found in secondary antibody repertoires. Affinity maturation by somatic hypermutation and receptor editing is likely to account for the amino acid sequence variation of JML-1, which revealed the strongest B-CLL cell surface reactivity, when compared to JML-3, -7, and -13. Although we cannot exclude the possibility that PCR errors and PCR cross-over artifacts contributed to this variation, a naïve human antibody library that we generated with the same reagents and protocols for selection on various antigens did not yield a comparable panel of highly homologues antibodies with identical LCDR3 and HCDR3 sequences. While our phage display approach involves random combination of heavy chain fragment and light chain, all selected Fab shared highly homologous chains with identical HCDR3 and LCDR3 sequences, suggesting that (i) both chains are necessary for antigen binding and (ii) that the selected pair pre-existed in the original post-alloHSCT antibody repertoire. It is thus tempting to speculate that JML-1 and its homologues contributed directly to GVL activity through tumor cell surface targeting as has been suggested for post-alloHSCT and post-DLI serum antibodies. In follow-up studies, we are investigating the antitumor activity of JML-1 IgG1, including induction of apoptosis in B-CLL cells and mediation of complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).

What do we know about the nature of the JML-1 antigen? The progressive elimination of B-CLL cells following alloHSCT could facilitate cross-presentation of a number of allogeneic antigens to the reconstituting immune system, and it is possible that at least some of the serum antibody reactivity we detected is directed against unmatched HLA (other than A, B, and DR; Table 1) of the alloHSCT recipient. Anti-HLA antibodies are frequently detected following organ transplantation and are believed to be involved in graft failure. Unlike an anti-HLA antibody, however, the dominant JML-1 clonotype showed B-cell lineage restricted reactivity against tumor and normal cells from a number of different B-CLL patients and healthy volunteers. In addition to its presence on the CD19+ subpopulation and its absence on the
CD19- subpopulation of PBMC, its absence on all B-cell lines tested excludes a number of cell surface proteins that are currently investigated as targets for mAb therapy of B-CLL and other B-cell malignancies, including CD5, CD19, CD20, CD22, CD23, CD25, CD32B, CD37, CD38, CD40, CD45, CD52, CD74, CD79A, CD79B, CD80, CD200, HLA-DR, surface Ig, and ROR1.4 Its distinct expression profile also excludes B-CLL cell surface proteins to which post-alloHSCT or post-DLI serum antibodies were previously discovered, such as OFA/iLR and BCMA.23,28 Notably, unlike SEREX, our methodology does not exclude non-peptidic epitopes, such as carbohydrates, lipids, and nucleic acids, which may or may not be associated with cell surface proteins. Preliminary experiments suggested that the JML-1 antigen is constitutively expressed by B-CLL cells, and activation of B-CLL cells by either soluble recombinant human CD40L protein plus recombinant human IL-4 or a mitogen (lipopolysaccharide) did not result in significant changes in the cell surface expression of JML-1 antigen on B-CLL cells or normal B cells from healthy volunteers (data not shown).

The identification of the JML-1 antigen is important for at least two reasons. First, it will allow to study whether the JML-1 antigen is a common target of post-alloHSCT and post-DLI serum antibodies as well as alloreactive donor T cells. Second, knowing the JML-1 antigen will help to define its expression profile in normal adult tissues and its possible expression in other cancers which are important criteria for the therapeutic potential of JML-1 IgG1. Our methodology provides recombinant Fab or IgG in infinite supply for a variety of antigen discovery strategies, including protein microarray screening, cDNA expression cloning, and protein sequencing following immunoprecipitation.

A key advantage of our strategy is the fact that it yields fully human mAbs. Due to their potentially lower immunogenicity fully human mAbs are considered superior to nonhuman, chimeric, and humanized mAbs in therapeutic applications that require repeated dosing.49 Post-alloHSCT antibody repertoires provide a novel route to the discovery of fully human mAbs. Expanding our strategy beyond B-CLL to other hematologic and solid malignancies that are treated with alloHSCT50 will determine its broader utility as an antibody drug and target discovery platform for cancer therapy.
Acknowledgements

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Authorship

Contribution: S.B. and C.R. designed and supervised the experiments, interpreted data, and edited the manuscript. S.B., J.M.S., and I.S. performed experiments and analyzed data. R.S., R.W.C., S.Z.P., and M.R.B. participated in the design of the study and interpretation of data and edited the manuscript. C.R. conceptualized the study and wrote the manuscript. The authors declare no competing financial interests.

References


Table 1. Characteristics of B-CLL patients treated with nonmyeloablative peripheral blood alloHSCT.

<table>
<thead>
<tr>
<th>Patient</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indication</td>
<td>Relapse</td>
<td>Relapse</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Age (^1)</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>Enrollment</td>
<td>2003</td>
<td>2002</td>
</tr>
<tr>
<td>Clinical trial (^2)</td>
<td>NCT00055744</td>
<td>NCT00003838</td>
</tr>
<tr>
<td>Induction chemotherapy (^3), (^4)</td>
<td>EPOCH + FR</td>
<td>FC</td>
</tr>
<tr>
<td>Donor (^5)</td>
<td>Brother</td>
<td>Brother</td>
</tr>
<tr>
<td>GVHD prophylaxis (^6)</td>
<td>CSP + MTX</td>
<td>CSP + MTX</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Response (^7)</td>
<td>PR; CR after DLI</td>
<td>CR</td>
</tr>
<tr>
<td>Current status (^8)</td>
<td>Molecular remission</td>
<td>Molecular remission</td>
</tr>
</tbody>
</table>

\(^1\) At enrollment; \(^2\) ClinicalTrials.gov identifier; \(^3\) EPOCH + FR, etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin plus fludarabine, rituximab; \(^4\) FC, fludarabine, cyclophosphamide; \(^5\) HLA-matched (6/6; A, B, and DR); \(^6\) CSP + MTX, cyclosporine plus methotrexate; \(^7\) PR, partial response, CR, complete response, DLI, donor lymphocyte infusion; \(^8\) May 2009.
Table 2. Cell surface reactivity of post-alloHSCT serum antibodies

<table>
<thead>
<tr>
<th></th>
<th>Autologous B-CLL cells</th>
<th>Autologous T cells</th>
<th>Allogeneic B cells</th>
<th>Third party B cells</th>
</tr>
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<tbody>
<tr>
<td><strong>Patient A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>post 6m plasma</td>
<td>++&lt;sup&gt;1&lt;/sup&gt;</td>
<td>+</td>
<td>-&lt;sup&gt;2&lt;/sup&gt;</td>
<td>weak</td>
</tr>
<tr>
<td><strong>Patient B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>post 10m plasma</td>
<td>++</td>
<td>weak</td>
<td>++&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>rituximab</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- (patient A)</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>+ (patient B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>alemtuzumab</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+++ (patient A)</td>
<td></td>
<td></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>+++ (patient B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Based on median fluorescence intensity (MFI) ratios calculated for sample over background (<2, “-“; ≥2 and <5, “weak”; ≥5 and <20, “+”; ≥20 and <100, “++”; ≥100, “+++”).

<sup>2</sup>Allogeneic B cells derived from PBMC of patient A fifteen months after transplantation.

<sup>3</sup>Allogeneic B cells derived from PBMC of the donor of patient B.
Table 3. Post-alloHSCT human Fab library.

<table>
<thead>
<tr>
<th></th>
<th>Post-alloHSCT PBMC</th>
<th>Normal PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Successful primer combinations</strong> $V_\kappa$</td>
<td>10/12 (83%)</td>
<td>12/12 (100%)</td>
</tr>
<tr>
<td><strong>Successful primer combinations</strong> $V_\lambda$</td>
<td>41/60 (68%)</td>
<td>59/60 (98%)</td>
</tr>
<tr>
<td><strong>Successful primer combinations</strong> $V_H$</td>
<td>81/114 (71%)</td>
<td>95/114 (83%)</td>
</tr>
<tr>
<td><strong>Total of successful primer combinations</strong></td>
<td>132/186 (71%)</td>
<td>166/186 (89%)</td>
</tr>
<tr>
<td><strong>Phagemid</strong></td>
<td>pC3C</td>
<td>not applicable</td>
</tr>
<tr>
<td><strong>Library size</strong></td>
<td>$2.6 \times 10^8$</td>
<td>not applicable</td>
</tr>
<tr>
<td><strong>E. coli strain</strong></td>
<td>XL1-Blue</td>
<td>not applicable</td>
</tr>
<tr>
<td><strong>Helper phage</strong></td>
<td>VCSM13</td>
<td>not applicable</td>
</tr>
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</table>
Table 4. Analysis of the amino acid sequences of selected human Fab.

<table>
<thead>
<tr>
<th>Light chain</th>
<th>JML-1</th>
<th>JML-3</th>
<th>JML-7</th>
<th>JML-13</th>
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</thead>
<tbody>
<tr>
<td>Human germlines(^1)</td>
<td>$V_\kappa$ 1-39</td>
<td>$V_\kappa$ 1-39</td>
<td>$V_\kappa$ 1-39</td>
<td>$V_\kappa$ 1-39</td>
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<tr>
<td></td>
<td>$J_\kappa$ 3</td>
<td>$J_\kappa$ 3</td>
<td>$J_\kappa$ 3</td>
<td>$J_\kappa$ 3</td>
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<tr>
<td>Deviation from $V_\kappa$ germline(^2)</td>
<td>0/95</td>
<td>2/95</td>
<td>1/95</td>
<td>3/95</td>
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<td>LCDR3 sequence</td>
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<td>QQSYPSTPT</td>
<td>QQSYPSTPT</td>
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<tr>
<td>Sequence identity to JML-1</td>
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<td>98%</td>
<td>99%</td>
<td>97%</td>
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<tr>
<td>Sequence identity to JML-3</td>
<td>98%</td>
<td>100%</td>
<td>97%</td>
<td>95%</td>
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<tr>
<td>Sequence identity to JML-7</td>
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<td>97%</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>Sequence identity to JML-13</td>
<td>97%</td>
<td>95%</td>
<td>96%</td>
<td>100%</td>
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<table>
<thead>
<tr>
<th>Heavy chain</th>
<th>JML-1</th>
<th>JML-3</th>
<th>JML-7</th>
<th>JML-13</th>
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<tbody>
<tr>
<td>Human germlines(^1)</td>
<td>$V_H$ 3-9</td>
<td>$V_H$ 3-30</td>
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<tr>
<td></td>
<td>$J_H$ 3</td>
<td>$J_H$ 3</td>
<td>$J_H$ 3</td>
<td>$J_H$ 3</td>
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<tr>
<td>Deviation from $V_H$ germline(^2)</td>
<td>7/98</td>
<td>2/98</td>
<td>2/98</td>
<td>1/98</td>
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<tr>
<td>HCDR3 sequence</td>
<td>GGQTIDI</td>
<td>GGQTIDI</td>
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<td>GGQTIDI</td>
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<td>89%</td>
<td>90%</td>
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<tr>
<td>Sequence identity to JML-3</td>
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<td>99%</td>
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<td>98%</td>
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<tr>
<td>Sequence identity to JML-13</td>
<td>88%</td>
<td>99%</td>
<td>98%</td>
<td>100%</td>
</tr>
</tbody>
</table>

\(^1\)Based on DNA alignments using IMGT/V-QUEST (http://imgt.cines.fr).
\(^2\)Based on amino acid sequence alignments using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast).
\(^3\)Based on amino acid sequences of the variable domains.
Figure Legends

**Figure 1. Development of a sensitive flow cytometry assay to detect the binding of human antibodies to primary B-CLL cells.** PBMC (5 x 10⁵, consisting of >85% B-CLL cells) from an untreated B-CLL patient were first incubated with normal goat serum to block Fcγ receptors (CD32B) followed by unconjugated goat Fab anti-human IgG pAbs to block cell surface Ig (“blocking”). In the pilot experiment shown here, chimeric mouse/human anti-human CD20 mAb *rituximab* was the primary antibody that served as positive control for serum antibodies with human constant domains and cell surface reactivity. Goat F(ab′)₂ anti-human IgG pAbs conjugated to Qdot 655 nanocrystals (“γ-h-Qdot”) were used as secondary antibodies. Substantial reactivity noted for the secondary antibody alone (A) was eliminated through cell surface blocking (B), permitting detection of the primary antibody reactivity (C). The numbers inside each panel depict median fluorescence intensity (MFI).

**Figure 2. Detection of post-alloHSCT human serum antibodies binding to primary B-CLL cells.** PBMC (5 x 10⁵, consisting of >85% B-CLL cells) harvested prior to induction chemotherapy from alloHSCT recipients patient A and B were incubated with plasma samples collected from each patient at the indicated time points. Plasma from alloHSCT donor (“donor”) or healthy volunteers (“control”) was included. The assay procedure described for Figure 1 was followed. Using a CD3-FITC/CD19-PE two-color reagent, PBMC were gated into B cells (dominated by B-CLL cells) and T cells. B-cell (black) and T-cell surface reactivity (white) is depicted as MFI for patient A (left panel) and patient B (right panel). Wk, weeks; m, months.

**Figure 3. Generation and selection of a post-alloHSCT human Fab library by phage display.** (A) Using a total of 186 different and separate combinations, human Vκ, Vλ, and VH encoding sequences were amplified by RT-PCR from post 6m PBMC derived from patient A, assembled into Vκ-Cκ-VH and Vλ-Cλ-VH cassettes by 3-fragment overlap extension PCR, and cloned into phagemid pC3C by asymmetric SfiI ligation. The resulting post-alloHSCT human Fab library was subsequently converted from phagemid to phage by transformation of *E. coli* and addition of helper phage. (B) The post-alloHSCT human Fab library was selected by three
rounds of panning on PBMC derived from untreated B-CLL patient α (round 2, round 3, and round 5). Two additional rounds of panning on immobilized rat anti-HA mAb were carried out (round 1 and round 4). The steps of one cell panning round are shown.

**Figure 4. Enrichment of phage displaying human Fab.** (A) Phage output-to-input ratios increased approximately 50-fold over the three cell panning rounds indicating the enrichment of phage displaying human Fab with cell surface reactivity. (B) Polyclonal phage from the three cell panning rounds were analyzed for cell surface reactivity in a whole cell ELISA using PBMC derived from untreated B-CLL patient α and mouse anti-phage mAb conjugated to horse radish peroxidase as detecting antibody. Shown are signals after subtraction of the signal obtained for the detecting antibody alone.

**Figure 5. Selective binding of JML-1 IgG1 to primary B-CLL and B cells.** (A) B-CLL PBMC (5 x 10^5) were incubated with 1 μg/mL biotinylated JML-1 IgG1 or 1 μg/mL biotinylated TT11 IgG1, and subsequently stained with CD19-APC to allow gating of B cells. In a parallel experiment, MACS-separated CD19+ and CD19- subpopulations of PBMC from a healthy volunteer were used. Shown are flow cytometry profiles of B-CLL PBMC from patient α (used for library selection; upper left), from patient A (the alloHSCT recipient from whom the library originated; lower left), and CD19+ and CD19- subpopulations of PBMC from a representative healthy volunteer (upper right and lower right, respectively). The histograms reveal the binding of biotinylated JML-1 IgG1 (green) and biotinylated isotype control TT11 IgG1 (red) detected by PE-coupled streptavidin. The background signal obtained for the detection reagent alone is shown in black. (B) PBMC from fourteen B-CLL patients (including patient α and patient A), PBMC subpopulations from eleven (CD19+) and three (CD19-) healthy volunteers, and eleven B-cell lines (including B-CLL cell lines EHEB and 232-B4) were analyzed for JML-1 IgG1 and TT11 IgG1 binding as described above. Each data point depicts the MFI of an individual sample minus the MFI obtained for the detection reagent alone. Horizontal lines indicate arithmetic mean values; p, probability based on Mann-Whitney test.
Fig. 1

Fluorescence intensity

Number of events

Blocking
Rituximab
Gah-ODot

CD20
Y
CD32B

200

10^1
10^2
10^3
10^4
10^5

1027
18.3
835.4

B-CLL
Fig. 2

A

Patient A

- CD3-CD19+
- CD3+CD19-

B

Patient B

- CD3-CD19+
- CD3+CD19-

MFI

Control  Donor  Pre  Post1wk  Post1m  Post3m  Post6m  Post1yr  Post3yr  Post5yr  Post10yr
Fig. 3

A

B

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Fig. 4
**Fig. 5**

**A**

- B-CLL PBMC (Patient α)
- CD19+ PBMC (Healthy volunteer)
- B-CLL PBMC (Patient A)
- CD19+ PBMC (Healthy volunteer)

**B**

- Number of events
- Fluorescence intensity
- $\Delta$ MFI
- $p=0.023$

- B-cell lines
- B-CLL PBMC
- CD19+ CD19-
- Healthy volunteer PBMC
A human monoclonal antibody drug and target discovery platform for B-cell chronic lymphocytic leukemia based on allogeneic hematopoietic stem cell transplantation and phage display

Sivasubramanian Baskar, Jessica M. Suschak, Ivan Samija, Ramaprasad Srinivasan, Richard W. Childs, Steven Z. Pavletic, Michael R. Bishop and Christoph Rader