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A Novel Method Using Blinatumomab for Efficient, Clinical-Grade Expansion of Polyclonal T Cells for Adoptive Immunotherapy

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Current treatment of chronic lymphocytic leukemia (CLL) patients often results in life-threatening immunosuppression. Furthermore, CLL is still an incurable disease due to the persistence of residual leukemic cells. These patients may therefore benefit from immunotherapy approaches aimed at immunoreconstitution and/or the elimination of residual disease following chemotherapy. For these purposes, we designed a simple GMP-compliant protocol for ex vivo expansion of normal T cells from CLL patients’ peripheral blood for adoptive therapy, using bispecific Ab blinatumomab (CD3 × CD19), acting both as T cell stimulator and CLL depletion agent, and human rIL-2. Starting from only 10 ml CLL peripheral blood, a mean 515 × 10⁶ CD3⁺ T cells were expanded in 3 wk. The resulting blinatumomab-expanded T cells (BET) were polyclonal CD4⁺ and CD8⁺ and mostly effector and central memory cells. The Th1 subset was slightly prevalent over Th2, whereas Th17 and T regulatory cells were <1%. CMV-specific clones were detected in equivalent proportion before and after expansion. Interestingly, BET cells had normalized expression of the synapse inhibitors CD272 and CD279 compared with starting T cells and were cytotoxic against CD19⁺ targets in presence of blinatumomab in vitro. In support of their functional capacity, we observed that BET, in combination with blinatumomab, had significant therapeutic activity in a systemic human diffuse large B lymphoma model in NOD-SCID mice. We propose BET as a therapeutic tool for immunoreconstitution of heavily immunosuppressed CLL patients and, in combination with bispecific Ab, as antitumor immunotherapy.

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Chemotherapy of chronic lymphocytic leukemia (CLL), especially using fludarabine-based regimen, is limited by toxicity and the profound immunosuppression that ensues (1, 2). CLL patients also show several immune dysfunctions already at diagnosis, in particular defective T cells and T-mediated immune surveillance leading to increased susceptibility to infections (1, 3, 4). Ab therapy targeting Ags such as CD20 and CD19 has limited efficacy in CLL, and the use of alemtuzumab also leads to life-threatening immunosuppression (5, 6). More recently introduced drugs, such as kinase inhibitors ibrutinib and idelalisib, show promising therapeutic response, but are likely to benefit from further therapy with biological agents, including chimeric Ag receptor (CAR)-modified T cells or T cells combined with bispecific Ab, to eliminate minimal residual disease (7). For these reasons, CLL patients would greatly benefit from adoptive therapy with autologous normal T cells, expanded in vitro from the peripheral blood of patients before treatment is started, and frozen to constitute immunity after chemotherapy, or to be used as anti-tumor agents. Such expansions are, however, technically difficult because T cells represent generally only 1–30% of lymphocytes, whereas CLL cells are very abundant (50–99%).

In vitro T cell expansion protocols have been introduced for adoptive therapy in a number of contexts, including immune reconstitution, to expand tumor- or virus-specific cytotoxic T cells ex vivo for immunotherapy and to produce CAR-modified T cells (8–10). Most protocols rely on the use of anti-CD3 Ab on feeder layers or anti-CD3/CD28 microbeads, together with recombinant human IL-2 (rhIL-2). Starting T cells may be obtained from peripheral blood or from tumor tissue or adjacent lymph nodes, and either unpurified or immunoselected before expansion in vitro (8, 11, 12). However, these methods may favor the production of relatively differentiated and exhausted effector cells, with limited long-term persistence in vivo and in CLL, and removal of the abundant contaminating tumor cells is problematic. For these reasons, we have designed a novel GMP-compliant expansion protocol using the bispecific T cell–engaging Ab blinatumomab (CD3 × CD19, MT103, AMG103; Amgen, Thousand Oaks, CA). Blinatumomab cross-links CD3 on T cells and, in presence of CD19⁺ normal or neoplastic B cell targets to which its anti-CD19 portion binds, it activates them to proliferate and become cytotoxic to the B cell target (13–16). Thus, this single, clinical-grade reagent can be used for the simultaneous expansion of T cells and depletion of contaminating B and CLL cells.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BET, blinatumomab-expanded T cells; CAR, chimeric Ag receptor; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; PBMC, peripheral blood mononuclear cell; rhIL, recombinant human IL; Treg, regulatory T.

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Materials and Methods

Cells

The human cell lines BJAB (Burkitt’s lymphoma, CD19+), REH (pre-B-ALL, CD19+), and K562 (erythroleukemia, CD19+) were maintained in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (Euroclone, Wetherby, West Yorkshire, U.K.), 2 mM l-glutamine (Euroclone), and 110 μM gentamicin (PHT Pharma, Milano, Italy), supplemented with 0.4% methylcellulose (PCT Biotech, Milano, Italy). Normal human peripheral blood mononuclear cells (PBMC) were prepared from CLL patients or normal volunteers after informed consent by standard Ficoll Hypaque.

Expansion of T cells using blinatumomab and rhIL-2

To expand T cells, freshly isolated PBMC from untreated CLL patients or normal donors were plated at 3 × 10⁶/ml in serum-free X-VIVO 15 medium (Lonza) containing 0.1 mM gentamicin (FisioPharma, Palomonte, Italy), 10 ng/ml blinatumomab (AMG103; Amgen) (17, 18), and 500 IU/ml rhIL-2 (Bayer HealthCare Pharmaceuticals, Berlin, Germany). In two CLL cases, frozen PBMC were used. Every 2–4 d of culture, cells were counted in a Coulter counter (Beckman Coulter, Nyon, Switzerland) and expanded to a concentration of 1 × 10⁷/ml in fresh medium containing both blinatumomab and rhIL-2, as above, until complete disappearance of the B cells, after which only rhIL-2 was added. Cell products obtained after 18–24 d of culture were called blinatumomab-expanded T cells (BET).

Expansion of T cells using anti-CD3/CD28 microbeads and rhIL-2

PBMC from untreated CLL patients were plated at 3 × 10⁶/ml in serum-free X-VIVO 15 medium. Anti-CD3-CD28–conjugated magnetic beads (Dynabeads human T cell activator from Life Technologies) were added at a 3:1 bead/CD3+ cell ratio together with 500 IU/ml rhIL-2 and 5% human AB serum (Euroclone). Every 3–4 d of culture at 37°C, 5% CO₂, the cells were passaged to 1 × 10⁷/ml in fresh medium containing 500 IU/ml rhIL-2.

Immunofluorescence analyses by flow cytometry

The phenotypes of PMBC or expanded cultures were analyzed by direct immunofluorescence. Major subsets were analyzed using TCR-αβ FITC and TCR-γδ FITC, CD8 allophycocyanin or CD8 FITC, CD4 FITC or CD4 PE Abs, and CD62L PE and CD45RA FITC Abs (all from BD Biosciences, San Jose, CA) for naïve-memory subsets. To assess the presence of regulatory T (Treg) cells, cells were stained with CD4-FITC and CD25-PE Abs, permeabilized, and stained with anti-FOXp3 allophycocyanin Ab using the Foxp3 staining kit (BD Biosciences). For Th1, Th2, and Th17 evaluation, cells were treated for 5 h with PMA (50 ng/ml; Sigma-Aldrich, Milan, Italy) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of Golgistop Protein Transport Inhibitor (BD Biosciences). Cells were then fixed and permeabilized using Cytofix/Cytoperm solution and stained intracellularly using the CD4-Pacific Blue CS, IFN-γ FITC, IL-4 allophycocyanin, and IL-17A PE Ab mixture, according to the manufacturer’s instructions (Human Th1/Th2/Th17 Phenotyping kit; BD Biosciences).

For Vβ repertoire analysis, cells were stained using the Vβ repertoire kit from Beckman Coulter; CMV-specific T cells in starting or final cultures were identified by flow cytometry after staining with PE-conjugated HLA-A*0201/pp65495–503 tetramer (Immunotech Laboratories, Beckman Coulter, Marseille, France) and anti-CD8 FITC. The following Abs against markers of T cell activation/costimulation or inhibition were used to stain either the CD4+ or CD8+ subsets: anti-CD27 PE, anti-CD28 PE, anti-CD137 PE, anti-CD154 PE, anti-CD272 PE, anti-CD279 allophycocyanin, anti-CD244 PE, anti-NGK2D PE, anti-CD11a FITC, and anti-CD49 PE (all from BD Biosciences); anti-CD290R PE (eBioscience, San Diego, CA); and anti-CD152 PE (BioLegend, San Diego, CA). A FACSCANTO II flow cytometer (BD Biosciences) was used to analyze all samples in triple fluorescence.

Cytotoxicity assays

For cytotoxicity assays against target cell lines, cell lysis was measured by standard calcein release after 4-h incubation. For cytotoxicity measurements on CLL targets, expanded T cells were plated with CLL cells at a 3:1 or 1:1 E:T ratio and incubated overnight at 37°C, 5% CO₂. At the end of incubation, 50 μl calibration beads (Bright Count Microspheres; IQ Product, Groningen, the Netherlands) were added, and cells were then stained with CD19-FITC Ab and 7-aminoactinomycin D and analyzed on a FACSCANTO II Instrument. The absolute number of live CD19+7-aminoactinomycin D+ CLL cells was measured in each sample. Percentage cell lysis was then calculated using the following formula: 100 – (n of live CLL in test sample × 100/n of live CLL control sample).

In vivo immunotherapy

Lymphoma cells from a patient with diffuse large B cell lymphoma (DLBCL) treated with chemotherapy and rituximab were collected at relapse. These cells were inoculated i.p. in SCID mice (CB17/SCID; Charles River Laboratories, Calco, Italy) and were serially passaged in these mice for five passages, as previously described (19), and stored at <180°C. For immunotherapy experiments, passaged cells were then inoculated i.v. at 5 × 10⁶ per mouse in 5-wk-old female CB17.NOD/SCID mice (Charles River Laboratories). At days 6 and 20 (short schedule) or days 6, 20, 34, and 48 (long schedule), 20 × 10⁶ BET cells, expanded for 21 d from a normal donor, were inoculated i.v. with or without 100 ng blinatumomab. Due to the short t½ of blinatumomab, the same dose of Ab was inoculated i.v. also the 4 d following each treatment with BET plus blinatumomab or blinatumomab alone (13, 20). Animals were sacrificed when hind leg paralysis was observed and autopsied.

Statistical analysis

The data were analyzed with paired or unpaired Student t tests, as appropriate (*p < 0.05, **p < 0.01, and ***p < 0.001). For in vivo experiments, the Mantel–Cox method was used.

Results

Massive in vitro expansion of CD3+ cells from CLL patients using blinatumomab and rhIL-2

To generate large numbers of polyclonal T cells, we isolated PBMC from 18 untreated CLL patients. Median age was 67 y, and median time from diagnosis 7 mo; most were Binet stages A or B and Rai 0-II. None of the patients had received treatment prior to sample collection (Supplemental Table I). The freshly isolated (n = 16) or frozen PBMC (n = 2) were plated in serum-free medium in presence of 10 ng/ml blinatumomab and 500 IU/ml rhIL-2. Further blinatumomab and rhIL-2 were added again every 2–4 d for ~3 wk. Percentage and absolute numbers of CD3+ and CD19+ cells were measured by immunofluorescence at various times of culture. Expansion of CD3+ T cells with elimination of CD19+ CLL in 18–25 d of culture (median 22) was reproducibly obtained in all 18 cases. Fig. 1A and 1B show the complete time course obtained for 13 cases, and Table I reports the complete data of starting and final populations for all 18 expansions.

Starting material was a mean of 10.3 ml peripheral blood (range 2–30 ml). At the start of culture, PBMC contained a mean of 9.3% CD3+ cells (range 1.2–30%) and 79% CD19+ cells (range 53–98%). T cells rapidly expanded, becoming a mean 84.7% at the end of culture (Fig. 1A, Table I). The mean increase of T cells was 159-fold (range 4.4–996). Normalizing expansions to a starting volume of 10 ml, a mean of 515 × 10⁶ T cells (range 51–1285) could be obtained (Fig. 1B, Table I, column 9). In parallel with T cell growth, CD19+ CLL rapidly decreased and became generally undetectable between days 14 and 21 of culture, to reach a mean 0.2% at the end (Fig. 1A, 1C, Table I). Only in one case (BET23), a significant percentage (3.8%) of CLL B cells could still be observed at the end of culture (Fig. 1C, Table I), but this was due to the particularly high percentage of neoplastic cells in the starting population in this patient (98%), resulting in relatively slow depletion of these cells, which therefore remained detectable at day 24. Nonetheless, this patient sample showed a 42-fold expansion of T cells, from 3.6 × 10⁶ to 152 × 10⁶ (Table I).

We conclude that efficient and reproducible expansion of T cells and elimination of CLL cells can be achieved by the simple addition of blinatumomab and rhIL-2 to CLL PBMC cultures, without prior T cell enrichment. The expanded population was called BET.
**FIGURE 1.** Expansion of T cells from CLL patients using blinatumomab and rhIL-2. PBMC from 18 CLL patients were cultured with blinatumomab and rhIL-2. (A and B) For 13 expansions (BET3 to BET22 and BET24; see Table I), the percentages (A) and absolute numbers (B) of CD3+ and CD19+ cells were measured at different times by flow cytometry (time course), and these are shown as mean and SDs. (C) For all 18 expansions, the percentages of CD19+, CD3+, CD4+, and CD8+ cells were measured at the end of culture and are shown in (C), with each symbol representing a different expansion. Percentages of NK cells (CD56+CD3−) at the end of culture were measured only in the last 10 expansions (BET18 to BET29). The full details of the cell composition at start and end of culture are also shown in Table I.

**Phenotypic characterization of T cells expanded using blinatumomab and rhIL-2**

Phenotypic analyses of the final BET populations showed that, in most cases, CD3+ T cells were mixed CD4+ and CD8+ cells in variable ratios (mean 46% CD4+ and 44% CD8+). Only in two cases (BET3 and BET25), nearly pure CD4+ populations were obtained (94 and 95%, respectively) (Fig. 1C, Table I). The only significant contaminant was NK cells, and percentage of these cells at the end of culture was highly variable (mean 18.5%, range 0.2–56.7%) (Fig. 1C, Table I).

We also analyzed the TCR Vβ repertoire of CLL T cells pre- and postexpansion, to determine whether restricted clones of T cells are expanded in culture. In all four cultures analyzed, the expressed Vβ families were comparable on starting population of T cells and at the end of culture, and were within the range observed in normal donors (Fig. 2A). Furthermore, we could show that Ag-specific clones present at the beginning of culture were expanded by blinatumomab and rhIL-2. Indeed, in one HLA-A02+ patient, CD8+ T cells specific for CMV peptide pp65495–503 loaded onto the HLA-A*0201 tetramer were about 1% in both starting PBMC and final product (Fig. 2B). These data suggest that CLL T cells are expanded in a polyclonal fashion with blinatumomab and rhIL-2, without favoring specific clones.

Further phenotypic analyses revealed that the major subsets present within both the CD4+ and CD8+ populations were central memory and effector memory cells, which together represented >75% of BET at the end of culture (Fig. 3A). Furthermore, >95% of BET culture products expressed the αβ, but not γδ TCR, indicating that they are standard T cells (Fig. 3B). Finally, the Th1 subset was more represented in BET cells than the Th2, with a mean 20.5 and 10.5%, respectively, whereas Th17 cells could not be detected in significant amounts (<1%). Similarly, CD4+/CD25+/FOXP3+ Treg cells represented <1% of the final population (Fig. 3B).

We also analyzed expression in the CD4+ and CD8+ subsets of markers involved in cytotoxic activity (NKG2D), survival in vivo (CD27), costimulation (CD28, CD137, CD154), synapse formation (CD11a, CD49d), as well as molecules associated with T cell exhaustion or inhibition of activation/synapse formation (CD200R, CD244/SLAM, CD274/PDL1, CD272/BTLA, CD279/ PD1, and CD152/CTLA4). NKG2D was present on ∼90% of CD8+ BET and ∼10% of CD4+. Both CD4+ and CD8+ cells expressed high levels of CD27 (∼80 and 63%, respectively). Among costimulatory molecules, CD28 was well expressed, although at higher levels on CD4+ than CD8+ cells (95 and 33%, respectively), whereas CD137 and CD154 were not present to a significant extent on either subset. The synapse molecules CD11a and CD49d were well expressed on BET cells of both CD4+ and CD8+ populations (Fig. 3C). In contrast, BET expressed rather low amounts (1–20% positivity) of most inhibitory molecules, except CD200R, which was present in ∼41% of CD4+ and 22% of CD8+ cells (Fig. 3C).

**Normalization of the synapse inhibitory molecules CD272 and CD279 on CLL T cells during expansion**

Because CLL is known to be associated with functionally defective T cells that overexpress the synapse inhibitors CD272/BTLA and CD279/PD1 (21, 22), we analyzed expression of these markers. We first confirmed that CD272 and CD279 were expressed at higher levels in CLL T cells compared with normal donor T cells (Supplemental Fig. 1). Interestingly, both CD272 and CD279 were significantly downmodulated in BET-expanded cells compared with so-called Xcellerated T cells, expanded by guest on April 20, 2017 http://www.jimmunol.org/ Downloaded from
Table I. Details of 18 T cell expansions from CLL patients

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<th>Exp. No.</th>
<th>Starting Material</th>
<th>Volume (ml)</th>
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<th>% CD4</th>
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<th>% CD8</th>
<th>Fold Increase</th>
<th>% CD19</th>
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*aAbsolute CD3 numbers were normalized on 10 ml starting blood volume.*
with anti-CD3/28 beads. Both methods, performed in parallel starting from the same CLL samples, led to a reproducible and efficient T cell growth in culture, with gradual disappearance of CLL B cells during time (Fig. 4A, 4B). However, contaminating B cells decreased more rapidly in BET compared with Xcellerated cultures, as can be expected because blinatumomab not only expands T cells, but also induces B cell–specific cytotoxicity. This difference was significant at days 7 and 11 (Fig. 4A, 4B) (p < 0.05). Both BET- and CD3/CD28-expanded cultures contained a variable proportion of CD4+ and CD8+ cells and measurable contamination with NK cells (Fig. 4C). Similarly, we observed that Xcellerated cells showed a very similar phenotype to that of BET

FIGURE 2. BET are polyclonal and contain virus-specific clones. (A) The polyclonality of BET CD3+ cells was analyzed before (pre) and after (post) expansion by flow cytometry of TCR Vβ families (BET18, 20, 21, and 24). The data are the means and SDs of the percentage expression of TCR Vβ families for the four CLL cases pre-expansion (thick striped bars) and postexpansion (thin striped bars), compared with the minimal (black bars) and maximal (gray bars) values for a large panel of normal donors, as provided by the manufacturer. (B) PBMC from a HLA-A2+ CLL patient (BET18) positive for CMV was analyzed pre- and postexpansion using FITC-conjugated CD8 Ab and PE-conjugated HLA-A0201 tetramer loaded with the CMV pp65495–503 peptide and flow cytometry. The dot plots and percentages of HLA-A*0201/pp65495–503 tetramer-positive cells among CD8+ cells, pre-expansion and postexpansion, are shown.

FIGURE 3. Characterization of expanded CD4+ and CD8+ BET cells, and normalization of CD272 and CD279 synapse inhibitors. (A–C) PBMC from CLL patients were cultured with blinatumomab and rhIL-2, and the complete phenotype of the CD4+ (black bars) and CD8+ subsets at the end of culture was analyzed by flow cytometry. (A) The mean percentages of naive (N; CD45RA+, CD62L+), central memory (CM; CD45RA–CD62L–), effector memory (EM; CD45RA–CD62L–), and fully differentiated effector memory RA+ cells (EMRA; CD45RA–CD62L–) in the CD4+ or CD8+ subsets are shown (n = 5). (B) The mean percentages of TCR-αβ–positive or TCR-ββ–positive cells in the CD4+ and CD8+ subsets are shown. In addition, the mean percentages of CD4+ Th1, Th2, Th17, and FOXP3+ Treg cells are shown (n = 5). (C) The expression of a panel of markers of cytotoxicity (NKG2D), long-term in vivo persistence (CD27), costimulatory activity (CD28, CD137, CD154), synapse/cellular adhesion (CD11a, CD49d), and inhibitory activity (CD200R, CD244, CD274, CD272, CD279, CD152) was analyzed. (D) and (E) Percentage expression of CD272 and CD279 on CD4+ cells (D) and CD8+ cells (E) pre- and postexpansion was analyzed by flow cytometry. The results are the means and SDs of at least four different CLL samples. *p < 0.05, **p < 0.01 comparing values post- versus pre-expansion.
for most markers analyzed (Supplemental Fig. 2). The major
difference observed was lower CD27 expression in CD3/CD28-
expanded T cells compared with BET (∼36% compared with 73–
83%, respectively, p, 0.01) (Fig. 4D). In contrast, the other co-
stimulatory molecule CD28 was expressed at high levels, especially
on CD8+ cells of both populations (Fig. 4D).

**BET cells show antitumor activity in presence of blinatumomab
in vitro and in vivo**

To test the functionality of BET, we first analyzed their cytotoxic
capacity against CD19+ cell lines or CLL cells in vitro. In presence
of blinatumomab, BET cells showed significant cytotoxic activity
against the CD19+ cell lines BJAB and REH (53–58%), even at
a low 3:1 E:T ratio (Fig. 5A). Cytotoxicity against cell lines was
comparable with that observed using CD3/CD28-expanded T cells
(Fig. 5B). BET were also highly cytotoxic against primary CLL
cells in presence of blinatumomab, with mean 43% lysis at the
very low 1:1 E:T ratio (Fig. 5C).

We finally wished to analyze the therapeutic potential of BET
in vivo in combination with blinatumomab. To do this, we chose
a CD19+ DLBCL model in which cells isolated from a patient
with DLBCL, who had relapsed after chemoimmunotherapy,
were expanded in vivo by five passages in SCID mice (19). The
BET cells used for the in vivo treatments were expanded from
a single normal donor for each experiment, because peripheral
blood was not available from the DLBCL patient. Indeed, T cells
from normal donors expanded with blinatumomab and rhIL-2
showed a similar phenotype as BET from CLL patients and, in
presence of blinatumomab, were cytotoxic to the same extent
toward CD19+ targets (Supplemental Fig. 3 and data not shown).
Therefore, to test the therapeutic potential of BET in vivo, 5 × 10^6
in vivo passaged DLBCL cells were inoculated i.v. in NOD-SCID
mice. At 2-wk intervals, groups of animals received either two
treatments (short schedule, starting days 6 and 20) or four treat-
ments (long schedule, starting days 6, 20, 34, and 48) of 20
× 10^6 BET, with or without 100 ng blinatumomab. Blinatumomab or

![FIGURE 4.](image-url) Comparison between BET and anti-
CD3/CD28–expanded T cells from CLL patients. PBMC from four CLL patients were expanded in
parallel for 23 d using (A) blinatumomab and rhIL-2
(BET), or (B) anti-CD3/CD28 microbeads and
rhIL-2. The percentages of CD3+ and CD19+ cells
at various times were analyzed by flow cytometry.
(C) At the end of BET (black bars) or CD3/CD28
microbead cultures (striped bars), the percentages of
CD4+, CD8+ T cells, and NK cells (CD3−CD56+)
were analyzed by flow cytometry. (D) The expression
of memory/costimulatory markers CD27 and CD28
on final BET (black bars) or CD3/CD28-expanded
T cells (striped bars) was measured on both the CD4+
and CD8+ subsets. **p < 0.01 comparing CD3/CD28
expanded versus BET cells.

![FIGURE 5.](image-url) BET cells are cytotoxic for CD19+ cell
lines and CLL targets in presence of blinatumomab. (A)
BET cells were used in cytotoxicity assays against the
CD19+ lymphoma/leukemia cell lines BJAB and REH,
at a 3:1 E:T ratio in presence (striped bars) or absence
(black bars) of 10 ng/ml blinatumomab. The results are
the means and SDs of seven (BJAB) and four (REH)
experiments. (B) BET at the end of culture were used as
effector cells at 3:1 and 1:1 in cytotoxicity assays
against freshly isolated CLL targets in presence (striped
bars) or absence (black bars) of 10 ng/ml blinatumomab.
Cytotoxicity was measured by flow cytometry. The
results shown are the means and SD of five experiments.
(C) CD3/CD28 expanded T cells were used in cytotoxic-
ity assays against BJAB and REH targets (E:T 3:1, n =
2 for each cell line), in presence (striped bars) or absence
(black bars) of 10 ng/ml blinatumomab. The p values
refer to the difference in presence versus absence
of blinatumomab. **p < 0.01, ***p < 0.001.
FIGURE 6. BET have therapeutic activity in presence of blinatumomab in a DLBCL tumor model. A total of $5 \times 10^6$ in vivo passaged DLBCL–PER cells was inoculated i.v. on day 1 in groups of NOD-SCID mice. (A) Treatment schedules: at days 6 and 20 (short schedule) or at days 6, 20, 34, and 48 (long schedule). $20 \times 10^6$ BET cells from a single normal donor for each experiment (colored arrows) were administered i.v. either alone or with 100 ng blinatumomab (black arrows). Blinatumomab was also daily administered i.v. for 4 d following each BET injection. (B and C) Survival curves of animals treated according to the short (B) or long (C) schedule.

Discussion

We report a novel GMP-compliant protocol, which makes use of the dual capacity of blinatumomab to induce both T cell proliferation and B cell depletion, for the expansion of autologous normal T cells from CLL patients for immune reconstitution purposes and immunotherapy.

Culture of PBMC from untreated CLL patients in serum-free medium containing blinatumomab and rhIL-2 led to a reproducible T cell expansion in $\sim 3$ wk, even in cases in which T cells in the starting population represented only 1–2% of PBMC. Expansion did not require prior T cell enrichment, a significant advantage for a GMP-compliant method, and used only clinical grade reagents. CLL B cells disappeared between days 7 and 14 and were undetectable by flow cytometry at the end of culture in most cases. A mean $515 \times 10^6$ T cells could be obtained starting from only 10 ml peripheral blood from untreated CLL patients. Thus, performing the same expansion on a larger scale for adoptive transfer and assuming a starting blood volume of 250 ml, a quite feasible quantity, it is expected that $\sim 1 \times 10^{10}$ autologous T cells would be obtained, corresponding to $\sim 140 \times 10^6$ T cells/kg. This value compares well with previous clinical data on the use of in vitro expanded T cells for immune reconstitution purposes (12, 23–26). The only major contaminant of BET cultures was NK cells. The percentage of NK cells at the end was highly variable and seemed to be patient dependent. NK cells are, however, not expected to be of negative impact for the intended use of adoptive transfer of BET.

Several reports suggest that CLL patients’ peripheral T cells may in some cases be oligoclonal or have skewed TCR Vβ usage (27, 28). The blinatumomab-expanded cells in this study were polyclonal and contained Ag (CMV virus)-specific clones that were regularly expanded along with other clones. Thus, BET cultures are likely to contain T cells with a variety of specificities. BET were composed of both CD4+ or CD8+ cells, although the proportion of each of these subsets was variable between cultures, with 2 of 18 containing nearly pure CD4+ cells. The presence of CD4+ cell is important for full CD8+ functionality. BET cells were skewed preferentially toward the Th1 rather than Th2 phenotype and did not contain a significant number of proinflammatory Th17 or immunosuppressive Treg cells. The latter point is important because contamination of even a relatively small percentage of Treg has been shown to significantly reduce the immune response in a number of contexts (29, 30). Final products had mostly a central memory and effective memory phenotype within both helper and cytotoxic T cell subsets. These results are consistent with previous reports showing induction of effector memory CD4+ and CD8+ T cells following blinatumomab treatment in vitro and in vivo (18, 31, 32). Interestingly, BET cells expressed CD27, the receptor for CD70, in vivo (18, 31, 32). The blinatumomab-expanded cells in this study were also demonstrated to be capable of activating and expanding CD4+ T cells in vivo (18, 31, 32). Finally, the inhibitory molecules CD244 (2B4), CD274 (PDL1), CD272 (BTLA), CD279 (PD1), and CD152 (CTLA4) were either very low or absent from the surface of BET. Most interestingly, the inhibitory molecules CD244 (2B4), CD274 (PDL1), CD272 (BTLA), CD279 (PD1), and CD152 (CTLA4) were either very low or absent from the surface of BET. Recent data have shown that freshly isolated CLL T cells express high levels of the synapse regulators CD272 and CD279 and defective synapse formation compared with normal T cells (22, 42–44), in part explaining the poor T cell–mediated immune response in these patients (21, 45, 46). We confirmed in this study that CD272 and CD279 are more highly expressed on T cells from CLL patients compared with normal donors. In addition, we showed that, during BET cultures, the high levels of these markers were normalized to the basal levels found on normal T cells, in both the CD4+ and CD8+ subsets. This downmodulation may be due to the rapid elimination of CLL cells during culture induced by blinatumomab and/or the positive stimulatory signal given by the bispecific Ab through CD3, overcoming possible negative interactions between CLL and T cells. It is worth noting that CD272 and CD279 have been strongly implicated as attenuators...
of the immune response and tumor tolerance and, for this reason, are prime targets for immunotherapy (47–52).

We also compared our expansion protocol with the Xcellerated CD3/CD28 method. The efficiency of T cell expansion and final cell phenotype was similar with both methods. However, blinatumomab has the unique property to stimulate T cell proliferation and at the same time induce cytotoxicity against CLL cells, explaining the earlier and complete CLL depletion observed in BET compared with CD3/CD28 cultures. This property is particularly useful for expansion in presence of a high CLL burden, because the method does not require prior T cell selection, an important aspect for large-scale and GMP-compliant protocols.

Finally, CD27, a marker of long-term survival, was more highly expressed on BET cells compared with CD3/CD28 Xcellerated T cells.

In confirmation of the functionality and effective cytotoxic synapse formation induced by BET cells, they were highly cytotoxic in vitro in presence of blinatumomab against CD19+ neoplastic primary B cells as well as cell lines, even at low E:T ratios. Finally, the antitumor potential of BET was demonstrated in vivo on multiple occasions to provide blood samples for these studies.

Acknowledgments
We thank patients and normal donors who have generously agreed on multiple occasions to provide blood samples for these studies.

Disclosures
D.N. is an employee of Amgen, the firm producing blinatumomab. The remaining authors have no financial conflicts of interest.

References

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The Journal of Immunology
Supplementary Table I. CLL Patients characteristics

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Supplementary Figure 1. Expression of CD272 and CD279 in normal donor and CLL T cells. Upper panel: CD4+ cells; lower panel: CD8+ cells
Supplementary Figure 2. Phenotype of CD3/CD28 expanded T cells from CLL patients.
Supplementary Fig.3. Expansion of BET from PBMC of normal donors.

(A) Absolute number of CD3⁺ T cells pre- and post-expansion are shown; mean fold expansion for 3 experiments was 120. (B) Percentages of T cells, CD4⁺, CD8⁺, CD19⁺ B cells and NK cells before and after expansion.