Blinatumomab induces autologous T-cell killing of chronic lymphocytic leukemia cells

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ABSTRACT

Chronic lymphocytic leukemia is an incurable B-cell malignancy that is associated with tumor cell-mediated T-cell dysfunction. It therefore represents a challenging disease for T-cell immunotherapeutics. The CD19/CD3 bi-specific antibody construct blinatumomab (AMG103 or MT103) has been tested clinically in non-Hodgkin's lymphoma and acute lymphoblastic leukemia but has not been assessed in chronic lymphocytic leukemia. We investigated whether blinatumomab could overcome T-cell dysfunction in chronic lymphocytic leukemia *in vitro*. Blinatumomab was tested on peripheral blood mononuclear cells from 28 patients (treatment naïve and previously treated). T-cell activation and function, as well as cytotoxicity against leukemic tumor cells were measured. Blinatumomab induced T-cell activation, proliferation, cytokine secretion and granzyme B release in a manner similar to that occurring with stimulation with anti-CD3/anti-CD28 beads. However, only blinatumomab was able to induce tumor cell death and this was found to require blinatumomab-mediated conjugate formation between T cells and tumor cells. Cytotoxicity of tumor cells was observed at very low T-cell:tumor cell ratios. A three-dimensional model based on confocal microscopy suggested that up to 11 tumor cells could cluster round each T cell. Importantly, blinatumomab induced cytotoxicity against tumor cells in samples from both treatment-naïve and treated patients, and in the presence of co-culture pro-survival signals. The potent cytotoxic action of blinatumomab on tumor cells appears to involve conjugation of T cells with tumor cells at both the activation and effector stages. The efficacy of blinatumomab *in vitro* suggests that the bi-specific antibody approach may be a powerful immunotherapeutic strategy in chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and accounts for 25-30% of newly diagnosed leukemias. It is a lymphoproliferative disease characterized by the clonal expansion of $CD19^+CD5^+CD23^+$ B cells in the bone marrow, blood and secondary lymphoid organs.[1] CLL has a heterogeneous clinical course with a spectrum of diseases ranging from indolent disease not requiring treatment to aggressive disease that is fatal.[2] At present there are no curative therapies for CLL, with the possible exception of allogeneic stem cell transplantation,[3] but this option has high morbidity and mortality rates, and is only suitable for a small minority of patients.

Over the last 20 years monoclonal antibodies, most notably alemtuzumab and rituximab, have emerged as new therapeutics in CLL.[4,5] The chimeric anti-CD20 monoclonal antibody rituximab has brought about significant improvements in response rates, progression-free survival and overall survival when used in combination with fludarabine and cyclophosphamide in previously untreated patients.[6,7] Alemtuzumab has been used as monotherapy as well as in combination with chemotherapies, high-dose steroids and other monoclonal antibodies and has proved to be extremely useful in patients with p53 mutations/deletions, as it appears to be cytotoxic via a p53-independent mechanism.[8] However, it is highly immunosuppressive[9] and so there is a need for newer less toxic, more effective and targeted therapeutic agents both for relapsed and treatment-naïve patients with co-morbidities.[10]

Blinatumomab is a bi-specific, single-chain antibody construct classified as a bi-specific T-cell engager (BiTE®). It is formed by the recombinant fusion of an anti-CD3 single-chain variable fragment (scFV) with an anti-CD19 scFV via a short peptide linker. These bi-specific antibodies can recruit immune effector cells to the tumor cell surface and promote immune synapse formation.[11] Blinatumomab has already shown encouraging clinical activity; in a phase II trial 80% of patients with relapsed acute lymphoblastic leukemia achieved a complete molecular response[12] and in a phase I study in relapsed non-Hodgkin's lymphoma (NHL) clinical responses were seen both in patients with indolent as well as aggressive B-NHL.[13,14] However, given the well-known T-cell dysfunction seen in CLL, blinatumomab may be less effective in this disease.[15-17]

In the current study, we provide a detailed analysis of the mechanism of action of blinatumomab-induced cytotoxicity in CLL. Our findings are consistent with a model that relies upon the sequential activation of T cells and CLL cells and demonstrate that blinatumomab retains efficacy even in the presence

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of CLL activation and pro-survival signals. Given these data, this approach represents a novel and promising therapeutic strategy for the treatment of CLL and, potentially, other lymphoid malignancies.

Methods

Blood samples from patients with chronic lymphocytic leukemia

Twenty-eight CLL patients (aged 53-83 years) were studied with full ethical approval. All blood samples from patients were obtained with informed consent (see *Online Supplementary Methods*). The majority of the assays were performed on fresh samples from treatment-naïve patients, except those for which the results are shown in Figure 1: in these assays, samples from patients who had received standard chemotherapy treatment were also tested.

Antibodies and flow cytometry

A full list of the antibodies used can be found in the *Online Supplementary Methods*. Blinatumomab (MT103, AMG103) was provided by Amgen Inc. (Munich, Germany). T cells were identified using CD4 and CD8 markers, while CLL cells were $CD5^+$ $CD4^-$ $CD8^-$ (>99% $CD19^+$).

Cell cultures

Peripheral blood mononuclear cells (PBMC) were incubated in RPMI 1640 supplemented with 5% AB serum (AB media) for 3-7 days. Blinatumomab was added to PBMC cultures at a concentration of 10 or 100 ng/mL. Human T-cell activator CD3/CD28 dynabeads (Invitrogen) were added at $1x10^5$ beads/$1x10^6$ PBMC as a positive control for T-cell activation. For absolute counts of T cells and CLL cells, an anti-CD3 antibody (Invitrogen) at a dose of 27.7 or 277 ng/mL was used as a positive control (same molar concentration as 10 or 100 ng/mL blinatumomab).

Intracellular Ki67 staining

PBMC were labeled with antibodies against CD4, CD8, CCR7 and CD45RA, fixed and permeabilized (Fix and Perm with 1% NP40, Caltag-Medsystems, Buckingham, UK) before staining with a Ki67-FITC antibody for flow cytometric analysis.

Cytokine secretion assay

Cytokines in tissue culture supernatants were measured using a Human Th1/Th2 11plex RTU Flowcytomix kit (eBioscience) for simultaneous detection of 11 cytokines.

Intracellular cytokine staining and the determination of CD107 and granzyme B expression

PBMC were cultured (3 days) with 10 ng/mL blinatumomab, before treatment with Golgi plug and Golgi stop (BD biosciences), and incubation with an anti-CD107 antibody (5 h at 37°C). Intracellular expression of granzyme B or interferon (IFN)-γ and tumor necrosis factor (TNF)-α in CD4 or CD8 T cells was measured by flow cytometry.

Absolute counts of T cells and chronic lymphocytic leukemia cells

PBMC samples that had or had not been treated with blinatumomab (7 days) were surface-stained with antibodies against annexin V, CD5, CD8 and CD4. Absolute counts were determined by flow cytometry using Cytocount beads (Dako, Stockport, UK).

Flow cytometry-based cytotoxicity assay

Mouse fibroblast cells transfected with CD40L [TL(CD40L)] and non-transfected cells (NTL) were cultured as previously described.[21] For co-cultures, irradiated (80 Gy) NTL or TL(CD40L) cells were seeded into a 48-well plate, and allowed to adhere before addition of CLL PBMC and blinatumomab (10 or 100 ng/mL) for 7 days. CLL PBMC were removed from the surface of the fibroblast monolayer, and analyzed by flow cytometry (annexin V, CD5, CD8 and CD4).

Immunofluorescence labeling for microscopy

Purified T cells and CLL cells were labeled with 15 mM red CFSE (Invitrogen) and and 10 mM green CFSE (Invitrogen) respectively. Labeled cells were put into culture at a ratio of one effector T cell to every ten target CLL cells for 12 h. Further details of phase contrast and confocal microscopy can be found in the *Online Supplementary Methods*.

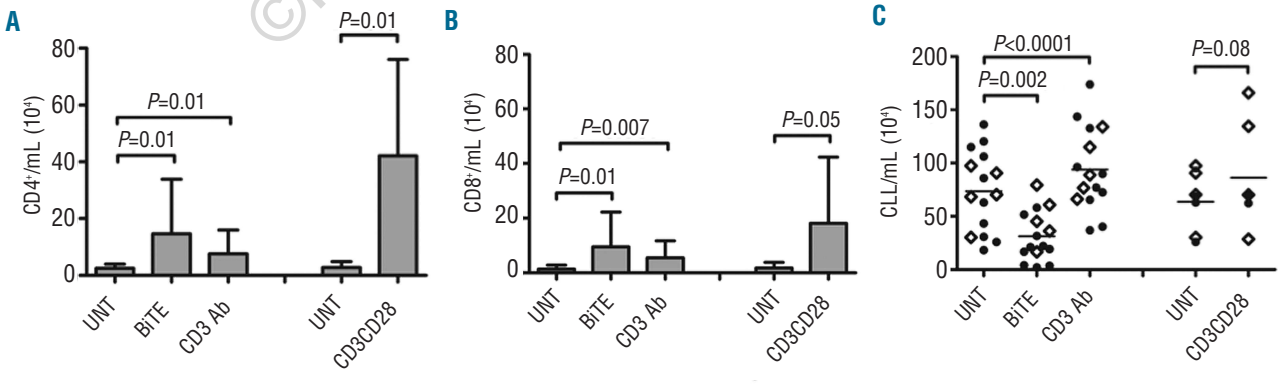

Figure 1. Blinatumomab mediates the expansion of T cells and a loss of CLL cells in PBMC cultures. Absolute numbers of $CD4^+$ T cells (A) and $CD8^+$ T cells (B) were calculated from CLL PBMC cultures after 7 days in the presence or absence of blinatumomab (10 ng/mL). Columns show the mean absolute number ± SD from 15 patients [BiTE® and CD3 antibody (Ab)] and 7 patients (CD3CD28). Mean starting numbers of $CD4^+$ and $CD8^+$ T cells were $6.9x10^4$ cells/mL and $4.1x10^4$ cells/mL, respectively. UNT=untreated, BiTE®= blinatumomab (10 ng/mL), CD3 Ab= anti-CD3 antibody and CD3CD28=CD3CD28 beads. Absolute numbers of CLL cells (C) were also determined in the same cultures. For this analysis, patients were divided into treatment-naïve (●) and treated patients (◊). Mean starting numbers of CLL cells in the treatment-naïve and treated patients were $135x10^4$ cells/mL and $113x10^4$ cells/mL, respectively.

Results

Blinatumomab induces the expansion of T cells and a reduction in chronic lymphocytic leukemia cells in primary leukemic samples

The effect of blinatumomab on PBMC cultures from 15 CLL patients was measured by determining absolute counts of T cells and CLL cells after 7 days. Both treatment-naïve (n=10), and previously treated (n=5) patients were tested. Cultures treated with blinatumomab had significantly higher absolute numbers of $CD4^+$ (P=0.01) and $CD8^+$ T cells (P=0.01) after 7 days when compared to untreated controls (Figure 1A and B). By contrast there was a significant reduction in the absolute numbers of CLL cells in the same cultures, suggesting a cytotoxic effect against leukemic cells (P=0.002; Figure 1C). In our system, spontaneous apoptosis of CLL cells averaged 45% (*data not shown*), however blinatumomab increased apoptosis to an average of 75% (*data not shown*, Figure 1C). This apparent cytotoxic effect on CLL cells did not differ between treatment-naïve and previously treated patients (Figure 1C).

Cultures treated with an anti-CD3 antibody (at the same molar concentration as blinatumomab) also demonstrated significant increases in absolute T-cell numbers (Figure 1A). However in sharp contrast to blinatumomab, the anti-CD3 antibody induced a significant increase in CLL cell numbers (Figure 1C). It is possible that CD3 triggering in the absence of co-stimulation is incapable of activating cytotoxicity against CLL, however cultures treated with anti-CD3/anti-CD28 beads showed similar results to those treated with CD3 alone; a significant increase in T cells (Figure 1A and 1B) and a trend towards increased CLL cell number (Figure 1C) after treatment. Interestingly in blinatumomab-treated cultures there was an inverse correlation between the change in T-cell numbers and percent change in CLL cell numbers (*Online Supplementary Figure S1A*) suggesting that the T-cell response was important to the cytotoxic effects observed. This was not the case in cultures treated with either anti-CD3 or anti-CD3/anti-CD28 (*Online Supplementary Figure S1B and C*); these cultures showed a trend towards increased T-cell numbers being associated with increased CLL numbers (*Online Supplementary Figure S1B and C*).

Taken together these results suggest that blinatumomab activates T cells in PBMC derived from primary CLL patients in a manner similar to anti-CD3 antibody or CD3/CD28 beads. However, unlike these stimuli, it appears that blinatumomab promotes T-cell-mediated cytotoxicity of CLL cells.

Blinatumomab induces death of chronic lymphocytic leukemia cells by apoptosis which is enhanced in the presence of CD40L

To confirm the cytotoxic effect of blinatumomab on CLL cells, PBMC cultures were treated with blinatumomab before measuring the numbers of annexin V^+ apoptotic CLL cells. Since CLL cells readily undergo apoptosis in liquid culture, it was possible that blinatumomab could be enhancing this process without any requirement for T cells. However initial experiments with purified CLL cells incubated with blinatumomab did not show any significant increase in apoptotic CLL cells (*Online Supplementary Figure S2*).

The effects of blinatumomab were additionally tested in a co-culture system with a CD40L transfected fibroblast cell line to promote cell survival.[18-20,22-24] In keeping with previous findings,[21] CLL cells were protected from apoptosis in the presence of both NTL and TL(CD40L) when compared to liquid culture (LC) alone (Figure 2A; NTL *versus* LC, P=0.02; TL(CD40L) *versus* LC, P=0.05). There was no significant difference in percent apoptotic CLL cells between the NTL and TL(CD40L) cultures. Despite the increased survival of CLL cells conferred by co-culture, blinatumomab increased the percentage of apoptotic annexin V^+ CLL cells under all three conditions tested when compared to untreated controls (Figure 2B). The percentage of apoptotic CLL cells induced by blinatumomab in the TL(CD40L) cultures was similar to that observed in LC (Figure 2B). Interestingly, there was a significant increase in the percentage of apoptotic CLL cells induced by blinatumomab when CD40L transfected fibroblasts were compared to non-transfected fibroblasts, suggesting that augmented CLL cell activation may even enhance the effects of blinatumomab (*Online Supplementary Figure S2*).

Blinatumomab induces T-cell proliferation and expansion of effector memory T cells

The expansion of $CD4^+$ and $CD8^+$ T-cell numbers in blinatumomab-treated cultures suggested that the bi-specific antibody could induce T-cell proliferation. This was confirmed by the significantly increased expression of the proliferation marker Ki67 in T cells ($CD4^+$ and $CD8^+$) after PBMC were treated with blinatumomab (Figure 3A and B).

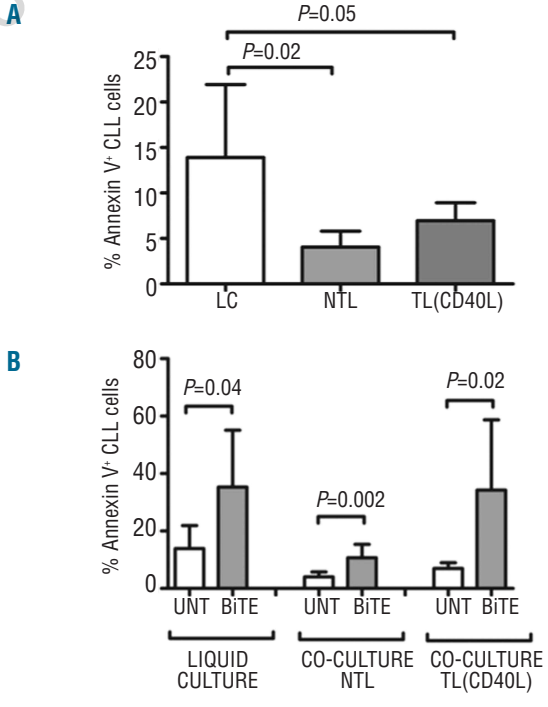

Figure 2. Blinatumomab-induced CLL cell death is maintained even in the presence of pro-survival signals. PBMC from CLL patients were incubated with blinatumomab (10 ng/mL) for 7 days in liquid culture (LC) or co-cultured on fibroblast cells transfected with CD40L, TL(CD40L). (A) The percentage of annexin V^+ CLL cells after incubation in LC or on non-transfected fibroblasts (NTL) or TL(CD40L). (B) The effect of blinatumomab (10 ng/mL) on the percentage of annexin V^+ CLL cells in LC or in co-culture with NTL or TL(CD40L). Columns show the mean ± SD (n=7). UNT =untreated BiTE®= blinatumomab (10 ng/mL). PBMC consisted of 87-96% CLL cells.

This increase in cycling T cells was evident on day 3, and increased further by day 7. Similar results were obtained using anti-CD3/anti-CD28 beads (Figure 3A and B). Furthermore, T cells from PBMC cultures treated with blinatumomab showed significant up-regulation of the activation markers CD38, HLA-DR and CD69 ($CD4^+$: *Online Supplementary Figure S4A,C,E* and $CD8^+$: *Online Supplementary Figure S4B,D,F*) by day 7. Blinatumomab treatment also resulted in increased expression of HLA-DR (*Online Supplementary Figures S5A and S4C*) on CLL cells, suggesting an activation effect. It appeared that this effect required the presence of T cells, as blinatumomab did not increase HLA-DR on purified CLL cells alone (*Online Supplementary Figure S5B*).

A previous study had demonstrated an expansion of T cells with an effector memory (EM) phenotype in a patient with non-Hodgkin's lymphoma who had responded to blinatumomab treatment.[16] It was of interest to determine whether blinatumomab had a similar effect on primary samples from CLL cells. Blinatumomab induced a significant increase in the percentage of EM $CD4^+$ T cells by day 7 when compared to untreated control cultures (Figure 3D). Concomitantly, the percentage of naïve $CD4^+$ T-cells decreased significantly (Figure 3C). Neither the EMRA or central memory (CM) $CD4^+$ T-cell population percentages were significantly altered compared to controls at day 7 (*Online Supplementary Figures S6A and S5B*). In the $CD8^+$ T-cell compartment, T cells predominantly displayed an EM and EMRA phenotype on day 0. Like the $CD4^+$ T cells, there was a significant increase in the percentage of EM T-cells in the $CD8^+$ T-cell compartment, this time evident at both day 3 and day 7 after blinatumomab treatment (Figure 3E). This increase in $CD8^+$ EM T cells coincided with a decrease in the percentage of $CD8^+$ EMRA T-cells at day 3 and day 7 (Figure 3F). Furthermore, the majority of $CD8^+$ EM T cells were still proliferating ($Ki67^+$) on day 7, whereas this was not the case for $CD8^+$ EMRA T cells (*Online Supplementary Figure S6E*). Both the naïve and central memory $CD8^+$ T-cell percentages remained relatively unchanged at both day 3 and day 7 (*Online Supplementary Figure S6C,D*). It is noteworthy that the capacity of blinatumomab to induce proliferation and activation of EM T cells was not unique, as similar patterns of response were also observed using the potent stimulus of anti-CD3/anti-CD28 beads (Figure 3A-F). However, as demonstrated in Figure 1, only blinatumomab had a cytotoxic effect on CLL cells.

Pro-inflammatory cytokines are released from peripheral blood mononuclear cell cultures treated with blinatumomab

To determine whether the difference in cytotoxic effect between blinatumomab and anti-CD3/anti-CD28 activated

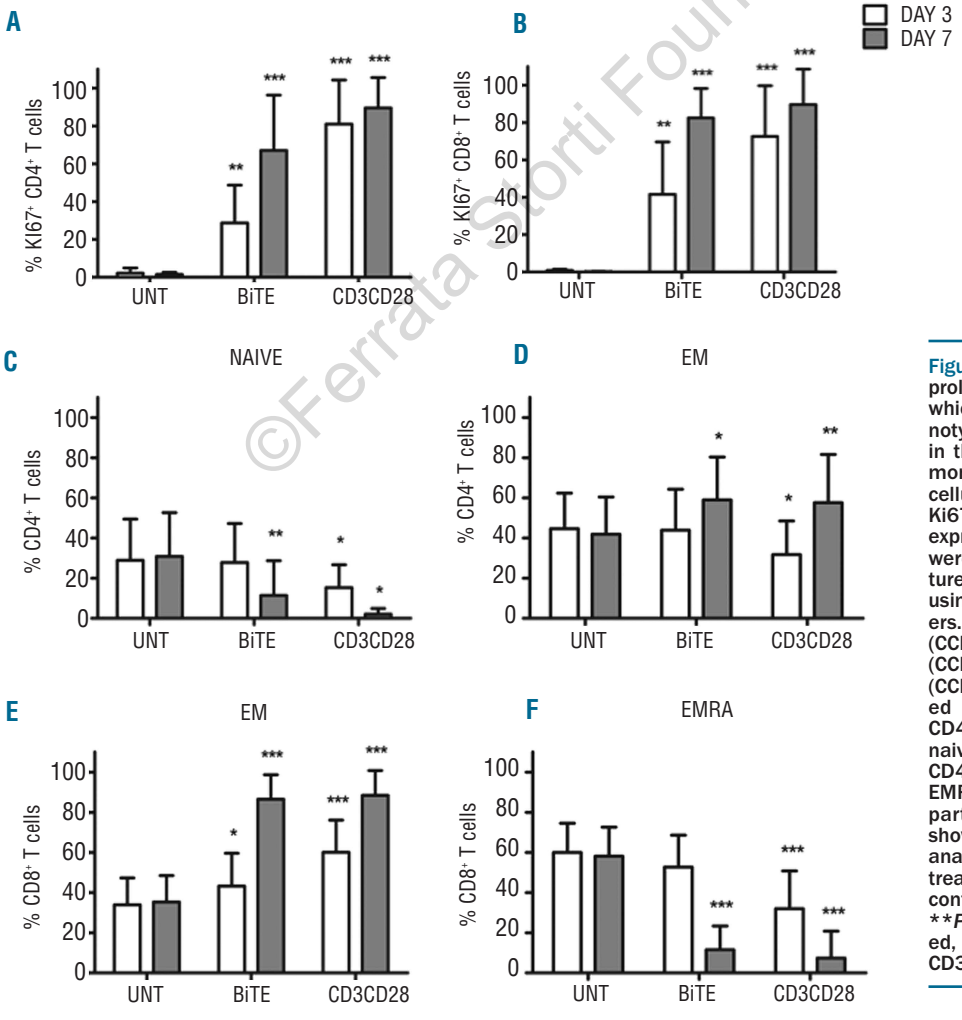

Figure 3. Blinatumomab induces the proliferation of T cells in CLL patients which are predominantly of an EM phenotype. T cells from CLL PBMC cultured in the presence or absence of blinatumomab (10 ng/mL) were stained intracellularly for the proliferation marker Ki67. (A-B) The percentages of Ki67 expressing $CD4^+$ (A) and $CD8^+$ (B) T cells were measured after 3 or 7 days in culture. T-cell subsets were differentiated using CCR7 and CD45RA surface markers. T-cell subsets were defined as naive ($CCR7^+CD45RA^+$), central memory (CM) ($CCR7^+CD45RA^-$), effector memory (EM) ($CCR7^-CD45RA^-$) and highly differentiated effector memory (EMRA) ($CCR7^-CD45RA^+$). (C-F) The percentages of naive (C) and EM (D) T cells within the $CD4^+$ compartment and EM (E) and EMRA (F) T cells within the $CD8^+$ compartment were measured. Columns show the mean ± SD (n=7). Statistical analysis was based on the results of treated cultures compared to untreated controls at day 3 or day 7. *P<0.05), **P<0.01, ***P<0.001. UNT=untreated, BiTE®= blinatumomab (10 ng/mL), CD3CD28=CD3CD28 beads.

T cells was cytokine-mediated, PBMC cultures treated with blinatumomab or anti-CD3/anti-CD28 beads were analyzed for the presence of 11 different cytokines. Three cytokines (IFN-γ, TNF-α and TNF-β) and one chemokine, interleukin (IL)-8, were found to be significantly increased in the supernatant after 3 days of culture with blinatumomab or CD3/CD28 beads (Figure 4A-D). With regards to anti-inflammatory or modulatory cytokines, no significant increases in IL-4, IL-5 and IL-10 were seen after treatment with blinatumomab (*data not shown*). Intracellular cytokine staining demonstrated that the cytokines detected in the supernatants were derived from T cells (Figure 4E and F).

T cells release cytotoxic granules and express high levels of interferon-γ and tumor necrosis factor-α after incubation with blinatumomab

No significant difference was seen in cytokine profiles of T cells activated by blinatumomab or anti-CD3/anti-CD28. It is, however, possible that the difference in cytotoxicity activity against CLL cells could be explained by differences in secretion of cytotoxic molecules or granules. Both blinatumomab and anti-CD3/anti-CD28 beads had the capacity to induce expression of granzyme B, when compared to untreated controls (Figure 5A,C). This was seen for both $CD4^+$ (Figure 5A) and $CD8^+$ T cells (Figure 5C). To assess whether the T cells were releasing this granzyme B, surface CD107 expression was measured as a marker of degranulation. The percentage of $CD4^+$ and $CD8^+$ T cells expressing both granzyme B and surface CD107 increased significantly after blinatumomab or anti-CD3/anti-CD28 bead treatment (Figure 5B,D).

Blinatumomab induces clustering of chronic lymphocytic leukemia cells around T cells

There was no obvious difference in the capacity of blinatumomab to induce T-cell activation based on several criteria (proliferation, cytokine release or cytotoxic granule release) when compared to anti-CD3/anti-CD28 beads. There was, however, a clear difference in cytotoxic effect against CLL cells. Blinatumomab-induced T-cell activation appeared to require the presence of CLL cells; proliferation of CD4 or CD8 T cells, and cytotoxic granule release of CD8 T cells was not induced in purified T-cell populations (*Online Supplementary Figure S7A-C*). Together these results suggested that physical interactions between T cells and CLL cells might be important for blinatumomab action.

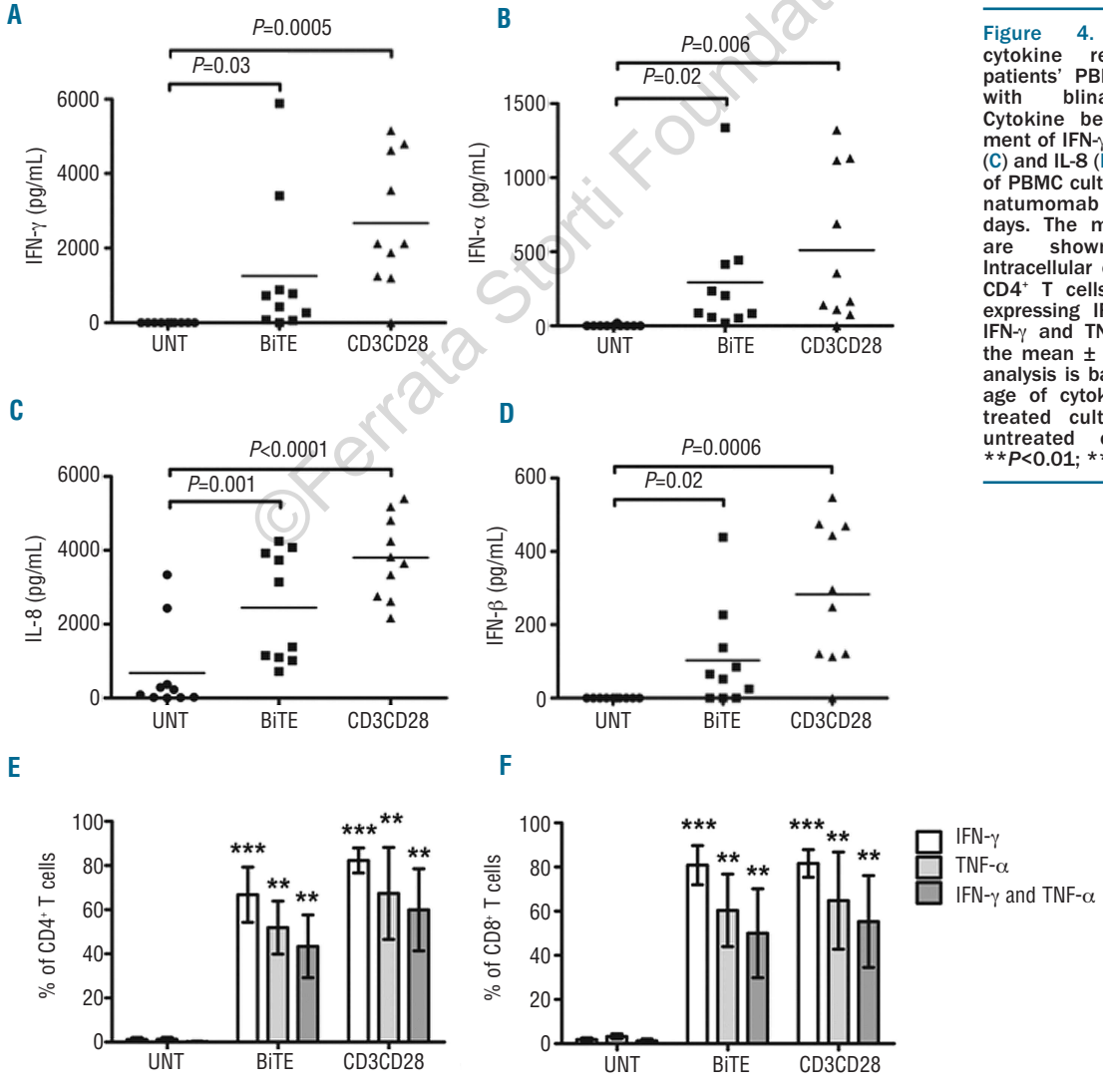

Figure 4. Pro-inflammatory cytokine release from CLL patients' PBMC after incubation with blinatumomab. (A-D) Cytokine bead array measurement of IFN-γ (A), TNF-α (B), TNFβ (C) and IL-8 (D) in the supernatant of PBMC cultures treated with blinatumomab (10 ng/mL) for 3 days. The mean concentrations are shown (n=10). (E-F) Intracellular cytokine staining for $CD4^+$ T cells and $CD8^+$ T cells expressing IFN-γ, TNF-α or both IFN-γ and TNF-α. Columns show the mean ± SD (n=4). Statistical analysis is based on the percentage of cytokine-positive cells in treated cultures compared to untreated controls. **P*<0.05; ***P*<0.01; ****P*<0.001.

This notion was supported by low power microscopy studies, which showed the formation of cell clusters after purified T cells and CLL cells were incubated with blinatumomab for 12 h (Figure 6A,B). These clusters were not observed in untreated controls (Figure 6A) or after anti-CD3 antibody (Figure 6A) or anti-CD3/anti-CD28 bead treatment (Figure 6A). Higher resolution fluorescence confocal imaging of these clusters showed single T cells (CFSE red) surrounded by multiple CLL cells (CFSE green) (Figure 6C). Computer-generated three-dimensional reconstruction of these clusters revealed one T cell closely interacting in a centralized position with 11 CLL cells (Figure 6D). These data suggest that the efficacy of blinatumomab against CLL cells *in vitro* is related to its capacity both to activate T cells and to force conjugation with CLL cells.

Discussion

Many studies have identified that T-cell numbers and function are altered in CLL.[16,19,22-25] The precise role that different T-cell subsets play in the immunodeficiency of CLL remains undetermined but functional studies have identified defects in immune synapse formation, co-stimulatory/accessory molecule expression and cytokine release.[15-17] Despite these problems, several T-cell-based therapeutic strategies have been tried in CLL including adoptive transfer of anti-CD3/anti-CD28 activated T cells,[26] T cells expressing chimeric antigen receptors, and vaccine therapy with dendritic cells pulsed with CLL-cell lysates.[27-29] The potential power of T cells to mediate therapeutic responses in CLL was demonstrated in a recent study using adoptive transfer of gene-modified (CD19 chimeric antigen receptor and 4-1BB) T cells.[28] Complete remission was demonstrated for a single patient, although this required prior leukapheresis and lymphodepleting chemotherapy. The combination of profound T-cell dysregulation in CLL and the technical challenges involved in adoptive therapy/genetic modification approaches have limited the widespread clinical application of immunotherapy.

In this study we collected detailed *in vitro* evidence that the bi-specific antibody blinatumomab, directed against CD3 and CD19, can activate and induce the proliferation of T cells from CLL patients *in situ*. By several criteria (Ki67 expression, cytokine secretion, cytotoxic granule formation), blinatumomab appeared to have comparable potency to anti-CD3/anti-CD28 beads in activating functional T cells. Our study showed that there was preferential expansion of an EM phenotype in both the $CD4^+$ and $CD8^+$ T-cell compartments, a phenomenon previously reported in NHL patients treated with blinatumomab.[13] Both $CD4^+$ and $CD8^+$ T cells had increased intracellular expression of granzyme B and the degranulation marker surface CD107. This suggests that both T-cell subsets can be redirected to become cytolytic against CLL cells in the presence of blinatumomab. Although cytotoxic $CD4^+$ T cells are relatively rare in healthy subjects,[30] an expanded population of $CD4^+$ perforin$^+$ T-cells with an EM phenotype has been described in CLL patients.[31]

Our study used 3- or 7-day cultures of PBMC treated with blinatumomab. While these assays allowed the investigation of the activating effects of blinatumomab on T cells, it is possible that our assays may have missed the rapid killing of CLL cells (within 4-24 hours) that has been demonstrated by previous studies.[32,33] This direct cytotoxicity did not require activation or proliferation of T cells, and involved caspase activation.[33] However even with the reported rapid effects of blinatumomab, we were able to show significant cytotoxicity against CLL cells at day 7 in the majority of patients tested.

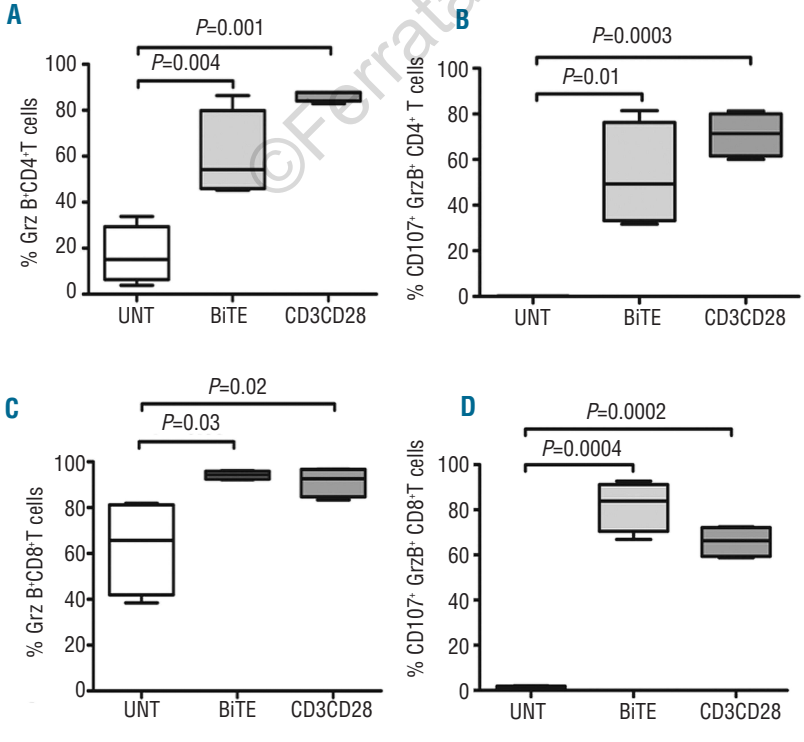

Figure 5. Blinatumomab increases the expression and release of cytotoxic granules from CLL patients' T cells. T cells from CLL PBMC cultured in the presence or absence of blinatumomab (10 ng/mL) were stained for intracellular granzyme B and surface CD107 expression. The percentages of granzyme B or granzyme B and CD107 expressing $CD4^+$ (A-B) and $CD8^+$ (C-D) T cells after 3 days in culture were measured. The means of each group are shown (n=4). UNT=untreated, BiTE®= blinatumomab (10 ng/mL), CD3CD28=CD3CD28 beads.

It is not clear whether the *in vivo* efficacy of blinatumomab[13] can be explained solely by the rapid tumor cell killing mechanisms demonstrated *in vitro*.[32,33] In the landmark study by Bargou *et al*. there were four complete responses in NHL patients after blinatumomab treatment.[13] These responses to blinatumomab were, however, variable; in two patients the tumor cells were not completely eliminated from the blood until day 24-30 of treatment (continuous infusion with blinatumomab), and in one of the patients approximately 10% annexin V^+ B cells could still be detected at day 24.[13] Blinatumomab-induced $CD8^+$ EM counts appeared to peak between days 7 and 18, with activated T cells peaking on day 18.[13] These results suggest that for some NHL patients, complete elimination of tumor cells from the blood requires the activation and expansion of effector $CD8^+$ T cells. For CLL, this might be most relevant for patients undergoing lymphodepleting chemotherapy, who will have low T-cell numbers after therapy. The rapid mode of killing induced by blinatumomab may be ineffective in these cases.[32] In our assay system, which measured the activation and expansion of T cells, the numbers of T cells were important; there was an inverse correlation between change in percent CLL cell numbers and change in CD4 and CD8 T-cell number (*Online Supplementary Figure S1*). Thus blinatumomab may have at least two modes of action *in vivo* and our study represents the first detailed characterization of T cells potentially involved in the second, slower mode of action.

We showed that an anti-CD3 antibody could also induce proliferation of T cells, but in contrast to blinatumomab caused an increase in CLL cell survival (P=0.02). This result is surprising given the poor survival of CLL cells in culture, but it is not unprecedented; Patten *et al.*[34] demonstrated that stimulation of T cells with anti-CD3/anti-CD28 beads increased the viability of CLL cells *in vitro*. In keeping with these findings, we showed that activation of T cells in PBMC cultures with anti-CD3/anti-CD28 beads led to the release of pro-inflammatory mediators including IFN-γ, TNF-α and IL-8, which are known to promote the survival of CLL cells *in vitro*.[35-37] Although similar cytokine release profiles were seen after blinatumomab treatment, in this scenario it was associated with significantly reduced survival of CLL cells. Our data do, therefore, suggest that T-cell activation and pro-inflammatory cytokine release are insufficient to induce CLL cell death. Rather, the T cell:CLL cell conjugation mediated by blinatumomab is a critical additional step in promoting the redirected secretion of cytotoxic molecules towards the surface of the tumor cell and ultimately CLL cell death. Although detailed investigation of immunological synapses was not performed in this study, it was possible that the bridging effect of blinatumomab was able to bypass the defects in immune synapse formation previously report-

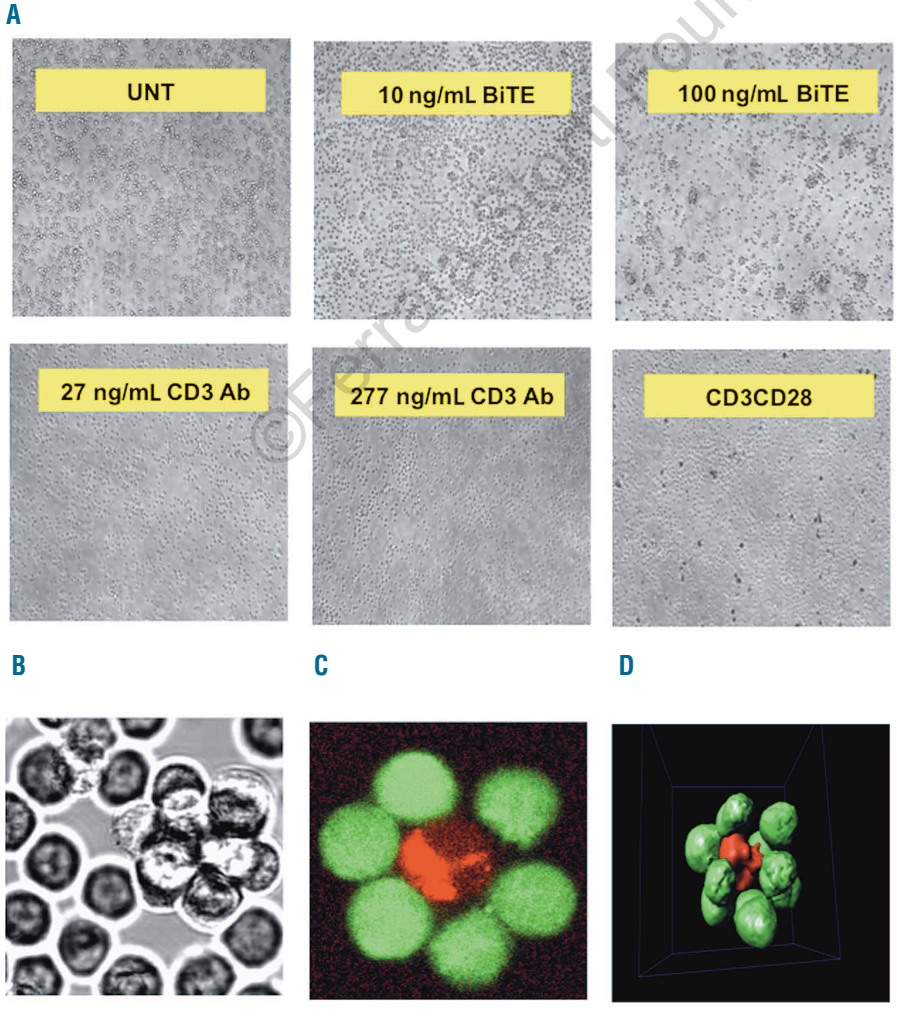

Figure 6. Visualization of CLL and T-cell clustering in the presence of blinatumomab. (A) CLL PBMC were incubated in culture for 12 h in the presence of BiTE® (10/100 ng/mL), and agonist anti-CD3 antibody (27 or 277 ng/mL) or CD3CD28 beads. T cells and CLL cells were stained with CFSE (red and green) and visualized after incubation for 12 h in the presence or absence of blinatumomab. (B) Cell cluster formations in cultures treated with blinatumomab (100 ng/mL). (C) Visualization of CLL cells (green) and a T cell (red) in a cluster by confocal microscopy. (D) Three-dimensional reconstruction of a cluster using Imaris imaging software; T cell (red) and CLL cells (green).

ed for CLL cells.[16] Our findings have potentially important implications for adoptive immunotherapy of CLL because they suggest that transferred T cells activated by anti-CD3/anti-CD28 or other polyclonal stimuli may not be effective without forced conjugation between T cells and CLL cells. It is also possible that blinatumomab could have an effect on tumor-specific T cells, by enhancing immune synapse formation between T cells and CLL cells with sub-optimal antigen presentation capacity.[16]

The efficacy of blinatumomab in our PBMC cultures is all the more remarkable because CLL cells can outnumber T cells by 40:1, creating very low effector:target ratios. Despite this, blinatumomab induced the formation of T-cell:CLL-cell conjugates analogous to those seen in conventional CTL:target interactions.[38] Importantly, this effect was not seen in cultures treated with an anti-CD3 antibody or anti-CD3/anti-CD28 beads, suggesting that the bi-specific nature of blinatumomab is crucial for the T-cell/tumor-cell conjugate formation and subsequent cytotoxicity of CLL cells.

There is growing appreciation that conventional chemotherapeutic agents have significantly less potency against CLL cells found in microenvironmental niches.[39,40] This is because microenvironmental signals, including interactions between CLL cells and CD40L-expressing T cells, stromal and nurse-like cells are known to increase the apoptotic threshold of CLL cells.[41,42] CD40-CD40L interactions have been shown to augment the antigen-presenting capacity of CLL cells due to increased surface expression of adhesion molecules (CD54) and co-stimulatory molecules such as CD80, CD86 and CD70 leading to CLL cell activation and protection from fludarabine-mediated killing.[18,43-45] Importantly, in this study we showed that CLL cells receiving activation signals through CD40-CD40L interactions in a co-culture model system remained susceptible to blinatumomab-mediated killing. Indeed, it would appear that increased expression of these molecules on the surface of CLL cells may even augment blinatumomab-directed T-cell activation and, ultimately, CLL cell killing (*Online Supplementary Figure S2*). These findings are in agreement with recent studies demonstrating the enhancing effect of CD40 stimulation on cytotoxicity mediated by rituximab[46] and GA101[47] antibodies. Five of the samples analyzed in this study were derived from patients with relapsed or refractory CLL with one patient also exhibiting a p53 deletion. It is of considerable interest that there was no significant reduction in the ability of blinatumomab to kill CLL cells from these patients when compared to chemo-immunotherapy naïve CLL cells (*data not shown*). These data indicate that the mechanism of action of blinatumomab is very different from that of other therapeutic agents and is probably p53-independent.

Our *in vitro* findings support a model in which blinatumomab promotes a forced conjugate between T cells and CLL cells (*Online Supplementary Figure S8*). This is consistent with previous studies on blinatumomab that have investigated mechanisms of cytotoxicity against lymphoma cell lines.[48] Similar observations demonstrating the cytotoxic effects of T-cell:CLL-cell conjugation have also been made using HLA/peptide/CD20 antibody constructs.[49] Since CLL cells are required for T-cell activation by blinatumomab (*Online Supplementary Figure S7*), it is likely that conjugate formation is required at both the activation and effector phases. We used confocal microscopy to generate a model in which 11 CLL cells can cluster around a single T cell. Moreover, we showed that T-cell activation is accompanied by CLL-cell activation, and that may render the CLL cells more susceptible to cytotoxicity. Importantly, the effector T cells generated can kill CLL cells even in the presence of pro-survival co-culture conditions.

In conclusion, blinatumomab has the ability to overcome the immunodeficiency in CLL *in situ* without the necessity to isolate the T cells from the tumor cells or to use gene transfer technology. Furthermore, it induces antigen-independent autologous T-cell activation *in situ* resulting in serial T-cell-mediated CLL cell killing. Given the recent reports of impressive clinical activity of blinatumomab in other B-cell neoplasms,[13,14,50] our data strongly support the clinical development of blinatumomab as a therapeutic agent in CLL.

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Authorship and Disclosures

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