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Targeting Human C-Type Lectin-like Molecule-1 (CLL1) with a Bispecific Antibody for Immunotherapy of Acute Myeloid Leukemia**

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Abstract: *Acute myeloid leukemia (AML), which is the most common acute adult leukemia and the second most common pediatric leukemia, still has a poor prognosis. Human C-type lectin-like molecule-1 (CLL1) is a recently identified myeloid lineage restricted cell surface marker, which is overexpressed in over 90% of AML patient myeloid blasts and in leukemic stem cells. Here, we describe the synthesis of a novel bispecific antibody, αCLL1-αCD3, using the genetically encoded unnatural amino acid, p-acetylphenylalanine. The resulting αCLL1-αCD3 recruits cytotoxic T cells to CLL1 positive cells, and demonstrates potent and selective cytotoxicity against several human AML cell lines and primary AML patient derived cells in vitro. Moreover, αCLL1-αCD3 treatment completely eliminates established tumors in an U937 AML cell line xenograft model. These results validate the clinical potential of CLL1 as an AML-specific antigen for the generation of a novel immunotherapeutic for AML.*

Acute myeloid leukemia (AML) is a disease that is characterized by the accumulation of rapidly proliferating and undifferentiated myeloid blasts in the bone marrow and the peripheral blood. Despite decades of clinical research, the five-year survival rate of AML is less than 30% for adult patients, regardless of receiving hematopoietic stem cell transplantation (HSCT).[1] A number of surface antigens, such as CD33, CD123, CD44, TIM-3, CD47, and CD32, have been explored as target antigens for AML treatment in the last few decades.[2] Some of these markers, however, can also be found in the healthy hematopoietic stem cell (HSC) compartment of normal bone marrow;[3] indeed, severe hematological toxicity was observed in targeted therapy utilizing an αCD33 antibody conjugated to calicheamicin (Mylotarg).[1,2,4] An ideal target for AML should therefore have minimum expression in the HSC compartment and on other normal cells for an improved therapeutic index. Human C-type lectin-like molecule-1 (CLL1, or CLEC12A), a recently identified myeloid lineage restricted cell surface marker, is an excellent therapeutic target for AML because of its overexpression in blasts and leukemic stem cells (LSCs)[5] in a majority (80–90%) of patients.[3b,6] Importantly, it has been reported that CLL1 expression is absent in the HSC compartment of normal and regenerating bone marrow.[3b,6d] Thus far, a monoclonal antibody that targets CLL1 has demonstrated therapeutic potential against AML cell lines through complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) mechanisms.[6a]

Bispecific antibodies that potently induce target-cell-dependent T cell activation have recently emerged as a successful immunotherapeutic approach for the treatment of cancer. Bispecific antibodies act through an amplified cytotoxic signal from tumor-localized activated effector T cells, making this an attractive approach for killing quiescent cancer stem cells, cancers overexpressing drug pumps, and cancer cells with low levels of surface antigens.[7] For AML, a bispecific antibody that targets the CD33 antigen, has previously been evaluated.[8] This bispecific antibody is selectively cytotoxic to AML cell lines and primary patient samples, and shows moderate efficacy in mouse xenograft models. However, other AML-associated antigens, such as the relatively novel marker CLL1, have yet to be successfully explored as targets for bispecific antibodies.

Previously, we reported an efficient method for generating bispecific antibodies (BiFabs) with defined geometries by conjugating two antigen-binding fragments (Fab) of antibodies that are site-specifically modified with bio-orthogonal chemical linkers.[9] Using this same method, we now report

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the synthesis of a novel CLL-1-targeting BiFab, αCLL1-αCD3, and a comparison of its in vitro and in vivo activity to a similarly constructed CD33-targeting BiFab, αCD33-αCD3. We show that although both BiFabs are cytotoxic toward AML cell lines and patient-derived cells, the αCLL1-αCD3 bispecific antibody has increased potency in vitro, and in contrast to αCD33-αCD3, completely eliminates established tumors in a subcutaneous xenograft mouse model using the human cancer cell line U937.

To synthesize αCD33-αCD3 and αCLL1-αCD3, we first expressed *p*-acetylphenylalanine (*p*AcF) mutants of αCD33 (clone hM195),[10] αCLL1 (clone 1075.7),[6a] and αCD3 (clone UCHT1) Fabs in *Escherichia coli* (*E. coli*).[11] Briefly, a *Methanococcus jannaschii* derived orthogonal amber suppressor tRNA and aminoacyl-tRNA synthetase (aaRS) pair, which was previously evolved to selectively incorporate *p*AcF into proteins, was co-expressed with Fab genes containing a TAG codon at position S206 (LC S206-*p*AcF αCD33) or S203 (LC S203-*p*AcF αCLL1) on the light chain, or K138 on the heavy chain (HC K138-*p*AcF αCD3) in the presence of *p*AcF. The molecular weight of each Fab mutant was verified by SDS-PAGE and ESI–MS (Table S1). Notably, the αCD33 and αCLL1 Fab mutants showed comparable binding affinities to their corresponding antigens as revealed by ELISA analysis; IC_{50} values of the αCD33 and αCLL1 Fab mutants are around 7.0 and 6.1 nM, respectively (Figure S1 in the Supporting Information). αCD33-αCD3 and αCLL1-αCD3 were generated according to a previously reported protocol[9b] (Figure 1 A, Table S1), purified by size-exclusion chromatography (GE Healthcare), and endotoxin was removed with Mustang Q membrane (Pall Corp.). The overall recovery yields of purified BiFabs were around 65 %. The size of the BiFabs was confirmed by SDS-PAGE gel (Figure 1 B), and the binding affinities of BiFabs to purified CD33 or CLL1 antigens were comparable to the αCD33 and αCLL1 Fab mutants as measured by ELISA, indicating that the conjugation reactions do not interfere with antibody–antigen interactions (Figure S1). Moreover, the αCD33 and αCLL1 Fabs selectively bind to the human cancer cell line U937 ($CD33^+$, $CLL1^+$, and $CD3^-$); αCD3 Fab binds only to human T cells ($CD33^-$, $CLL1^-$, and $CD3^+$), and αCD33-αCD3 and αCLL1-αCD3 bind both U937 and T cells with comparable affinity, as determined by flow cytometry (Figure 1 C,D).

To test the ability of αCD33-αCD3 and αCLL1-αCD3 to recruit effector cells and lyse target AML cancer cells, we developed a cell viability assay based on flow cytometry (detailed protocol available in the Supporting Information) to quantify the absolute numbers of viable AML cells in the presence of human peripheral blood mononuclear cells (PBMC). As shown in Figure 2 A, at an effector/target cell (E/T) ratio of 10:1, both αCD33-αCD3 and αCLL1-αCD3 potently lysed more than 90 % of U937 cells after 24 hours of incubation, with EC_{50} values of around 445 pM and around 41 pM, respectively; after 48 hours of incubation, the EC_{50} values were approximately 25 pM (αCD33-αCD3) and 2.1 pM (αCLL1-CD3), respectively. Similar potencies were observed with the HL-60 AML cell line ($CD33^+$ and $CLL1^+$) after 24 hours of incubation (Figure 2 B). In contrast, neither BiFab displayed any significant cytotoxicity to the $CD33^-$ and $CLL1^-$ human B-lymphoblast cell line, RS4;11 (Figure S2). Moreover, a negative control using a nonconjugated mixture of the corresponding antibodies failed to show any significant toxicity at concentrations up to 25 nM. To determine whether the cytotoxicity of the BiFabs is associated with antigen abundance on target cells, three human cell lines with different CD33 and CLL1 expression levels (U937: CD33 and CLL1 high; KASUMI-3: CD33 and CLL1 medium; and KG-1A: CD33 and CLL1 low; see Figure S3) were used to compare the cytotoxicities of BiFabs after an incubation period of 24 hours. Differential toxicities and potencies of both BiFabs were observed and correlated with the antigen expression levels of each cell line (Figure 2 C,D). Mechanistic studies indicated antigen-specific T cell activation and proliferation (Figures S4 and S5). Taken together, our data confirm that the BiFabs potently and selectively lyse target cells in an antigen-dependent manner. Notably, αCLL1-αCD3 is on average five times more potent than αCD33-αCD3 against all cell lines tested, underscoring the potential advantage of αCLL1-αCD3 as a therapeutic for AML.

Next, to provide more clinically relevant evidence for the therapeutic potential of the BiFabs, we tested the ex vivo toxicity of αCD33-αCD3 and αCLL1-αCD3 against samples derived from primary AML patients. PBMCs from seven AML patients (denoted as AML1-7, Table S2) were isolated using Ficoll den-

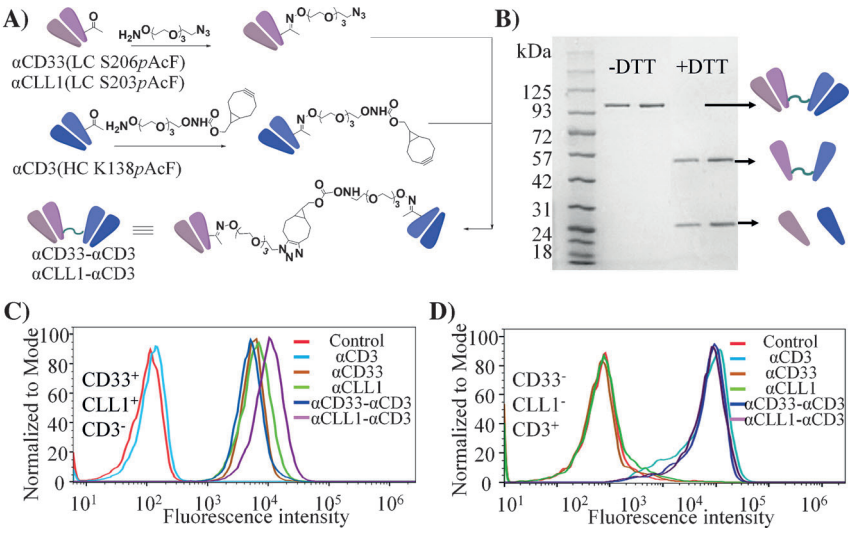

Figure 1. Synthesis and characterization of αCLL1-αCD3 and αCD33-αCD3. A) Synthesis of BiFabs. B) SDS-PAGE gel of BiFabs: lane 1 protein ladder; lanes 2–3 αCD33-αCD3 and αCLL1-αCD3, respectively, under nonreducing conditions; lanes 4–5 αCD33-αCD3 and αCLL1-αCD3, respectively, under reducing conditions. C,D) Binding of Fab mutants and BiFabs to human cell line U937 (C) and to human T cells (D); goat antihuman Kappa-RPE (Southern Biotech Associate) was used as the secondary antibody for cell labeling.

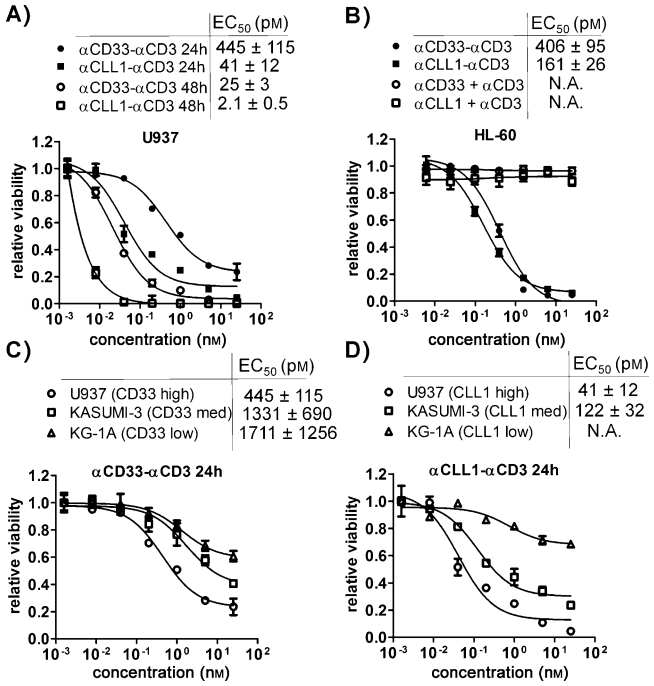

Figure 2. A,B) In vitro cytotoxicity of αCLL1-αCD3 and αCD33-αCD3 redirecting healthy PBMCs against various human AML cell lines, U937 (A) and HL60 (B), after 24 h or 48 h of incubation. C,D) Cytotoxicity curves of αCD33-αCD3 against U937, Kasumi-3 and KG-1A (C), and of αCLL1-αCD3 against U937, Kasumi-3 and KG-1A (D) after an incubation period of 24 h. In all experiments, target cells were stained by either PKH26 or CellVue Claret Far Red (Sigma–Aldrich) and incubated with PBMCs at a ratio of 1:10. Each data point represents a mean of triplicate samples. Error bars are representative of standard deviation. N.A. = not applicable.

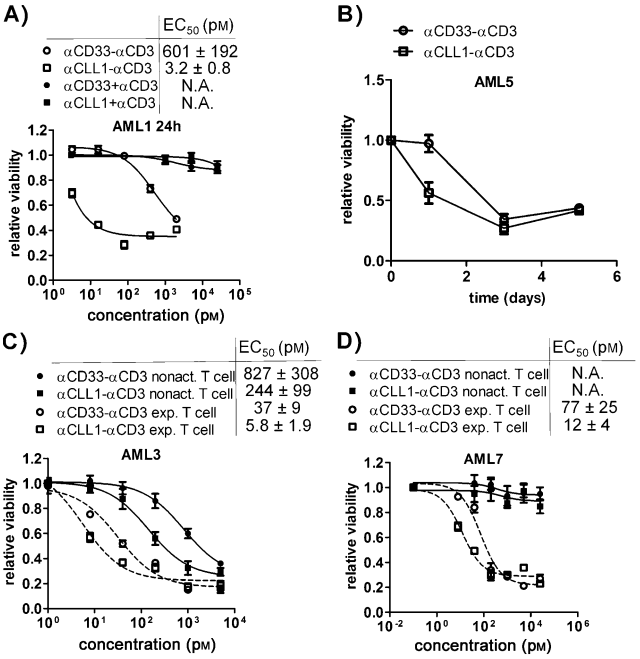

Figure 3. Ex vivo cytotoxicity of αCLL1-αCD3 and αCD33-αCD3 against primary AML patient samples. A) Relative viability of AML1 ($CD33^-$/$CLL1^+$) blasts treated with αCD33-αCD3, αCLL1-αCD3, nonconjugated Fab mixture of αCD33 + αCD3, or nonconjugated Fab mixture of αCLL1 + αCD3 for 24 h. B) Time-dependent ex vivo viability of AML5 ($CD33^+$/$CLL1^+$) blasts treated with 1 nM αCD33-αCD3 or αCLL1-αCD3. Relative viability of C) AML3 ($CD33^+$/$CLL1^+$) and D) AML7 ($CD33^+$/$CLL1^+$) blasts treated with αCD33-αCD3 or αCLL1-αCD3 with non-activated autologous T cells in PBMCs for 96 h (filled) or with ex vivo expanded autologous T cells for 48 h (open).

sity gradient centrifugation, and analyzed for subgroups of leukemic blasts,[12] T cells, and $CD33^+$/$CLL1^+$ cells (Table S2) by flow cytometry. Figure S6 depicts a representative gating scheme to identify blasts from a primary specimen (7-AAD^-/$CD34^+$/$CD45^{dim}$ or 7-AAD^-/SSC^{low}/$CD45^{dim}$). Flow cytometric analysis revealed that blasts in primary patient samples have differential expression levels of the CD33 and CLL1 antigens,[13] as determined by mean fluorescence intensity (MFI) values (see Table S2). Interestingly, among the seven primary samples, one (AML1) is $CD33^-$/$CLL1^+$, one (AML6) is $CD33^+$/$CLL1^-$, and the remaining five samples are $CD33^+$/$CLL1^+$.

Patient PBMCs were incubated in specialized serum-free medium (SFM) for a maximum of 6 days[14] with varying concentrations of BiFabs and monitored for cytotoxicity at different time points by flow cytometry. αCLL1-αCD3 induced satisfactory target cell lysis of AML1 ($CD33^-$/$CLL1^+$) blast cells within 24 hours at 3.2 pM and reached a plateau of approximately 72% blast killing at 80 pM (Figure 3A and Figure S7). However, αCD33-αCD3 showed poor cytotoxicity ($EC_{50} \approx 601$ pM) against AML1 blast cells, likely a consequence of the different CLL1 and CD33 expression levels (Table S2). In contrast, AML6 ($CD33^+$/$CLL1^-$) blast cells did not respond to a high concentration (25 nM) of αCLL1-αCD3 after 6 days of incubation (Figure S8), but showed modest cytotoxicity with αCD33-αCD3, confirming the target selectivity of BiFabs in primary patient samples. As for the five samples that are double-positive ($CD33^+$/$CLL1^+$), modest to excellent cytotoxicity (EC_{50} values ranging from 37–5170 pM, Table S2) was observed after 3–6 days of incubation with either αCLL1-αCD3 or αCD33-αCD3 (Figure S9–S11). Of note, although AML7 blast cells express both CD33 and CLL1 at high levels, this primary sample failed to respond to both BiFabs under our assay conditions (Figure 3D). The onset of blast cell death also differed among the samples. For instance, unlike the AML1 blasts which rapidly ($\approx$24 h) responded to αCLL1-αCD3 (Figure 3A), the AML5 blasts only showed detectable cytotoxicity after 24 hours, and reached a plateau after 72 hours of incubation (Figure 3B) with a maximum blast killing of 85% ($EC_{50} \approx 513$ pM) and 73% ($EC_{50} \approx 37$ pM) for αCD33-αCD3 and αCLL1-αCD3, respectively (Figure S11 and Table S2). In all instances, delayed or lack of responsiveness to BiFab treatment may potentially be attributed to the heterogeneity and/or suppressed T cell activity of primary samples.[13]

Considering the significantly lower proportion of T cells relative to blasts in AML patient samples (Table S2), and the high potential for suppressed T cell activity in these patients, we next tested whether BiFabs can redirect ex vivo expanded autologous T cell activity in the less responsive primary patient samples (i.e., AML2, AML3, AML4, AML6, and

AML7). Briefly, one vial of frozen patient PBMCs were thawed, activated by beads conjugated with αCD3/αCD28 antibody, and maintained with recombinant human IL-2 (rhIL-2) to enrich for mature T cells; 1–2 days prior to experimentation, cells were rested for 24–48 hours in SFM medium without rhIL-2. The ex vivo expanded T cells were then mixed with freshly thawed PBMCs from the same patient and seeded into a 96-well plate at indicated E/T ratios. In the case of AML3 blast cells at an E/T ratio of 1:1, cell lysis was observed in 24 hours, and reached a plateau of 85% (EC_{50} ≈ 37 pM) and 73% (EC_{50} ≈ 5.8 pM) blast killing after 48 hours with αCD33-αCD3 and αCLL1-αCD3, respectively (Figure 3C and Table S2). These results demonstrate an overall improvement in EC_{50} and induction time compared to the cytotoxicity of non-activated autologous T cells induced by BiFabs. Similar results were observed with activated T cells from AML2, AML4, AML6, and AML7 blasts (Figure S9, S10, S8, 3D, and Table S2). Interestingly, AML7 blast cells, which previously failed to respond to both BiFabs under native conditions, displayed excellent response when activated autologous T cells were used as effector cells with EC_{50} values of 77 pM and 12 pM at day 2 for αCD33-αCD3 and αCLL1-αCD3, respectively. However, even in the presence of expanded T cells, αCLL1-αCD3 treatment was ineffective against AML6 ($CLL1^-$) blast cells, confirming the excellent selectivity of αCLL1-αCD3 (Figure S8 and Table S2). Of note, nonspecific killing of blasts cells was not observed at an E/T ratio as high as 4:1 with the same expanded autologous T cells in the absence of BiFabs (Figure S12). Collectively, these results highlight the significance of functional T cells in this bispecific antibody approach (Figure 3D and Table S2). Finally, it should be noted that among the five AML patients that are both $CD33^+$ and $CLL1^+$ (AML2, AML3, AML4, AML5, and AML7), αCLL1-αCD3 showed higher potency than αCD33-αCD3 in four samples (AML3, AML4, AML5, and AML7, Table S2), which is in excellent agreement with previous in vitro killing results in AML cell lines.

The in vivo antitumor activity of the αCLL1-αCD3 was assessed in immunodeficient mice bearing tumors derived from human cancer cell line U937. U937 cells were inoculated into the subcutaneous flank of NOD/SCID/IL-2Rγ$^{-/-}$ (NSG) mice, and human PBMCs isolated from a healthy donor were injected intravenously (I.V.) into the mice on the same day. Xenografts were allowed to establish to an average size of 100–150 mm^3, after which human T cells that had been expanded in vitro were injected I.V. into the tumor-bearing mice. One day after the injection of the T cells, mice were injected I.V. with either the BiFabs or phosphate-buffered saline (PBS) daily for 10 days; the daily dosing schedule was based on the half-life (≈5 h) of similar BiFabs in mice (unpublished data). Tumors in PBS-treated mice showed rapid growth with a doubling time of approximately 2 days. In contrast, a significant reduction in tumor growth was observed in mice treated with BiFabs (Figure 4A). Moreover, the tumors in αCLL1-αCD3 treated mice steadily decreased in size and were barely detectable after 10 daily injections, demonstrating the ability of αCLL1-αCD3 to eradicate U937 tumor xenografts (Figure 3A). Single-cell preparations of residual tumors were analyzed by flow cytometry and

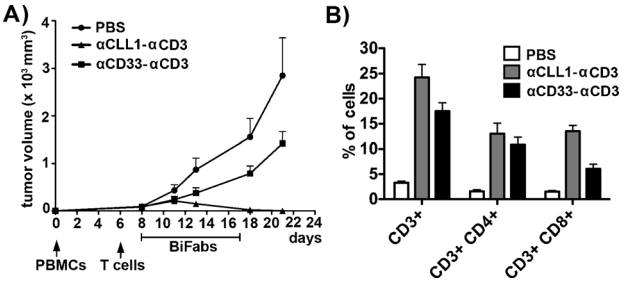

Figure 4. In vivo antitumor activity of αCLL1-αCD3 and αCD33-αCD3. A) U937 cells and PBMCs were injected in NSG mice on day 0. Upon observation of a palpable tumor, ex vivo expanded T cells were injected, and 24 h later, animals were dosed daily with αCLL1-αCD3 (1 mg kg^{-1}), αCD33-αCD3 (1 mg kg^{-1}), or PBS control for 10 days. B) T cell infiltrates in U937 tumors treated with αCLL1-αCD3 (1 mg kg^{-1}), αCD33-αCD3 (1 mg kg^{-1}), or PBS.

revealed the presence of intra-tumoral T cells in all BiFab-treated mice, whereas no significant T cell infiltration was observed in tumors from PBS-treated animals (Figure 4B). Additionally, no negative effects on the general health or body weights of the mice were observed in the BiFab-treated groups (Figure S13).

AML is a heterogeneous cancer with hierarchical cell populations that vary between individual patients, making it difficult to completely remove all malignant cells by a therapeutic agent that targets a single antigen. Indeed, analysis of AML CD33 and CLL1 expression in patients shows simultaneous expression in most patients, but cases of single antigen expression ($CD33^+/CLL1^-$ or $CD33^-/CLL1^+$) have also been reported (Ref. [6b]) and were observed in our AML patient samples. Therefore, we decided to generate an αCLL1-αCD3 bispecific antibody and compare it to a similarly generated αCD33-αCD3. αCLL1-αCD3 demonstrated potent and selective in vitro and in vivo cytotoxicity against various AML cell lines as well as ex vivo cytotoxicity against primary patient samples. Interestingly, αCLL1-αCD3 outperformed αCD33-αCD3 (despite similar apparent target binding affinities to their respective antigens) in all the AML cell lines we tested. However, it is worth pointing out that, given the heterogeneity of primary patient specimens, the efficacies of our BiFabs (and other T cell engagers) are determined by a multitude of parameters, including E/T ratio, antigen expression level, cytogenetics, and treatment history of patients. Nevertheless, this study demonstrates the potential of CLL1 as an AML target antigen, and our αCLL1-αCD3 as a promising T cell engaging bispecific agent. We are currently assessing the efficacy of αCLL1-αCD3 in additional primary samples and its ability to discriminate LSC and HSC populations in bone marrow samples from healthy donors and AML patients.

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