**In vitro and in vivo** model of a novel immunotherapy approach for chronic lymphocytic leukemia by anti-CD23 chimeric antigen receptor

**Running title:** CD23 CAR-targeting for CLL immunotherapy

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Abstract

Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of mature CD19⁺CD5⁺CD20dim B lymphocytes that typically express the B-cell activation marker CD23. Here we cloned and expressed in T lymphocytes a novel chimeric antigen receptor (CAR) targeting the CD23 antigen (CD23.CAR). CD23.CAR⁺ T cells showed specific cytotoxic activity against CD23⁺ tumor cell lines (average lysis 42%) and primary CD23⁺ CLL cells (average lysis 58%). This effect was obtained without significant toxicity against normal B lymphocytes, in contrast to CARs targeting CD19 or CD20 antigens, expressed physiologically also by normal B lymphocytes. Moreover, CLL derived CD23.CAR⁺ T cells released inflammatory cytokines (1445-fold more TNF-β, 20-fold more TNF-α and 4-fold more IFN-γ). Interleukin-2 was also produced (average release 2681 pg/ml) and sustained the antigen-dependent proliferation of CD23.CAR⁺ T cells. Redirected T cells were also effective in vivo in a CLL Rag2⁺/⁺γc⁻/⁻ xenograft mouse model. Compared with mice treated with control T cells, the infusion of CD23.CAR⁺ T cells resulted in fact in a significant delay in growth of MEC-1 CLL cell line. Altogether these data suggest that CD23.CAR⁺ T cells represent a selective immunotherapy for the elimination of CD23⁺ leukemic cells in patients with CLL.
Introduction

Chronic lymphocytic leukemia (CLL), the most common form of leukemia in adults in Western countries, remains an incurable disease despite the development of new therapeutic regimens. Allogeneic hematopoietic stem cell transplantation (HSCT) can be curative, but its application is limited to young adults who represent a small percentage of patients with CLL. Antibodies directed against different surface antigens are currently used in patients with CLL. Although anti-CD52 (Campath-1) antibodies rapidly reduce the leukemic burden in the peripheral blood, they have limited bio-distribution to secondary lymphoid organs, where CLL cells tend to accumulate. In the case of anti-CD20 antibodies, the low levels of the antigen on leukemic B cells limit their use as a single agent in this disease. In addition, antibodies do not lead to long-term control of the disease, since they do not establish an active memory immune response. CLL is also susceptible to cell-mediated immune control as indicated by the graft-versus-leukemia (GVL) effect associated with allogeneic HSCT, and by the immune responses elicited in patients receiving leukemia-tumor vaccines.

Adoptive transfer of T lymphocytes genetically modified to express a chimeric antigen receptor (CAR) can combine the beneficial effects of both antibody and T-cell mediated immune responses. CARs are chimeric molecules that contain an extracellular binding moiety derived from a monoclonal antibody (single chain variable fragment) (scFv) coupled to an intracellular signalling moiety (usually the ζ chain of the T-cell receptor complex). When expressed by T lymphocytes, CARs can trigger T-cell activation and perforin/granzyme-B release upon binding with the antigen expressed by tumor cells in a non MHC-restricted manner, thus avoiding an important mechanism of tumor immune-escape represented by the down regulation of MHC molecules by tumor cells. Adoptive transfer of CAR-transduced T lymphocytes may offer several advantages as compared to the passive administration of antibodies, since T cells have enhanced tissue bio-distribution and may establish a long-lasting anti-tumor immune response. CARs targeting either CD19 or CD20 antigens have been developed to treat human B-cell derived malignancies and clinical trials using these chimeric molecules are currently ongoing in several institutions. However, a potential major disadvantage of this strategy is represented by the fact that both CD19 and CD20 are expressed not only by leukemic cells, but also by normal B lymphocytes. Therefore, the sustained elimination of these cells by CAR-modified T cells could result in a severe impairment of the humoral immunity, exacerbating the characteristic immunodeficiency present in patients with CLL. With regard to the importance of preserving the normal B-cell compartment, the generation of CARs targeting antigens with a more restricted expression in tumor cells may have clinical relevance. CD23 antigen represents an attractive alternative in CLL, since leukemic B cells typically over express CD23, as compared to normal B lymphocytes. We have exploited this feature to selectively target malignant CLL cells, while sparing the normal B-cell compartment, by developing a novel CAR against this antigen.

Here we report that T cells engineered to express a CD23-specific CAR secrete immunostimulatory cytokines and have cytotoxic activity against CD23+ tumor cell lines and primary CLL cells in vitro, while...
spared normal B lymphocytes. Moreover, engineered T cells also provide a significant control of the leukemia growth in vivo, when infused in mice engrafted with MEC-1 CLL cell line, that closely reproduces the human CLL disease, and thus represents an optimal tool to test the efficacy of new therapeutic agents. In conclusion our data suggest that the administration of CD23.CAR’T cells may pave the way for a novel cell-based approach for the treatment of CLL.
Materials and methods

Cell lines and CLL primary cells
MEC-1 CLL cell line was obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). Wild type BJAB was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), while Jeko-1 Lymphoma B cell line was obtained from DMSZ. The latter two cell lines were genetically modified with a retroviral vector encoding the human CD23 molecule (PL-CD23-SP) to generate CD23⁺ BJAB and CD23⁺ Jeko-1 cells. Epstein-Barr virus (EBV)-transformed cell lines (LCLs) were obtained from the peripheral blood (PB) of healthy donors (HDs) as previously described. All cell lines were maintained in culture in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza, Bergamo, Italy), supplemented with 10% heat-inactivated foetal calf serum (FCS) (Lonza), 2 mM L-glutamine (Lonza), 25 IU/ml penicillin and 25 mg/ml streptomycin (Lonza) (complete RPMI). B lymphocytes were isolated from fresh PB mononuclear cells (PBMCs) of HDs using CD20 paramagnetic beads (Miltenyi Biotec, Calderara di Reno (BO), Italy), according to manufacturer’s instructions, while primary CLL cells were obtained from both fresh and frozen PBMC of CLL patients after removal of CD3⁺ T cells, using CD3 paramagnetic beads (Miltenyi Biotec), when T cells were >10%. Informed consent was obtained from both HDs and CLL patients according to protocols approved by the University of Milano-Biocca Institutional Review Board, following the Declaration of Helsinki.

Generation of activated T cells
PBMCs isolated by Ficoll (GE Healthcare, Milano, Italy) density gradient from PB collected from HDs and CLL patients were activated with 1 μg/ml anti-CD3 (OKT3 - eBioscience, San Diego, CA, USA) and 0.5 μg/ml anti-CD28 (Becton Dickinson, Milano, Italy) antibodies with the addition of 100 U/ml recombinant human IL-2 (Proleukin, Chiron Therapeutics, Emeryville, CA) in non-tissue culture treated 24well-plates (Becton Dickinson) at the concentration of 1x10⁶ cells/well.

Cloning of the CD23-specific CAR, generation of the retroviral supernatant and T-cell transduction
The sequence of the anti-human CD23 monoclonal antibody has been previously reported (accession numbers BD232452.1 and BD232451.1). Based on this sequence, we generated an optimized single chain antibody (scFv) encoding the variable regions of the heavy chain (VH) and light chain (VL) of the monoclonal antibody, using a synthetic DNA technology (Assembly PCR Oligo Maker). The scFv sequence was then cloned in frame with the human IgG1-CH₂CH₃ endodomain, the CD28 co-stimulatory endodomain and the ζ chain of the TCR/CD3 complex into the SFG retroviral backbone to generate the CD23.CAR vector as previously described. We used a CAR targeting the CD19 molecule, also containing the CD28 endodomain (CD19.CAR), as positive control vector. To produce the retroviral supernatant, 293T cells were co-transfected with CAR-containing retroviral vectors, Peq-Pam-e plasmid encoding the MoMLV gag-pol, and RDF plasmid encoding the RD114 envelope as previously described. Activated T
lymphocytes were then transduced with the retroviral supernatants using retronectin coated plates (Takara, Shuzo Co. Ltd, Shiga, Japan). After removal from the retronectin plates, T-cell lines were maintained in RPMI complete medium in a humidified atmosphere containing 5% CO\textsubscript{2} at 37°C with the addition of IL-2 (50 U/ml) every 3 days.

**Immunophenotyping**

Phycoerythrin (PE)-conjugated CD8, fluorescein isothiocyanate (FITC)–conjugated CD4, (PE)–conjugated CD56 and peridinin chlorophyll protein (PerCP)–conjugated CD3 monoclonal antibodies (mAbs) were used to stain T lymphocytes, whereas allophycocyanin (APC)-conjugated CD5, (FITC)-conjugated CD19 and (PE)-conjugated CD23 mAbs were used to stain tumor cell lines and primary CLL cells. All antibodies, except for CD8 and CD4 (Tema Ricerca S.r.L., Bologna, Italy), were purchased from Becton Dickinson. To detect the expression of CD19.CAR and CD23.CAR, T lymphocytes were stained with the Fc-specific cyanine-Cy5-conjugated (Fc-\(\gamma\)Cy5) antibody (Jackson ImmunoResearch, West Grove, PA), which recognizes the IgG1-CH\textsubscript{2}CH\textsubscript{3} component of the CARs. We evaluated the presence of regulatory T cells (Tregs) using (PercP)-conjugated CD4 (Becton Dickinson), (APC)-conjugated CD25 and (PE)-conjugated FOXP3 (Biolegend, Milano, Italy), and analyzed cells by fluorescence-activated cell sorting with a FACSscan flow cytometer (Becton Dickinson).

**Cytotoxicity assay**

Cytotoxic activity of control non transduced T cells (NT) and CAR\textsuperscript{T} T cells was measured using a standard 51\textsuperscript{Cr} release assay after a 4-hour incubation. The cell targets tested included Jeko-1, BJAB, and LCLs that were labelled with 25\(\mu\text{Ci}\) of 51\textsuperscript{Cr} (Perkin-Elmer Italia S.p.a, Monza, Italy) for 45 minutes, as well as primary CLL cells and normal B lymphocytes, each labelled with 50\(\mu\text{Ci}\) 51\textsuperscript{Cr} for 1 hour. 5x10\textsuperscript{3} target cells were then co-cultured in triplicate with T cells at Effector:Target (E:T) ratios 40:1, 20:1, 10:1 and 5:1, in complete medium alone or in 1% SDS (Sigma-Aldrich, Milano, Italy) to determine spontaneous and maximum 51\textsuperscript{Cr} release, respectively. Samples were collected and the mean specific lysis was calculated as previously described. While LCLs, CD23\textsuperscript{+} Jeko-1 and CD23\textsuperscript{+} BJAB maintain surface CD23 expressed when the cells are kept in culture, in primary CLL cells CD23 is cleaved after few hours of in vitro culture, largely due to the lack of specific plasmatic metalloprotease-inhibitors in the cell culture medium. Therefore, in order to evaluate the CD23.CAR-specific killing of primary CLL cells, we pre-incubated leukemic lymphocytes with the metalloprotease-inhibitor Imidazole (Sigma-Aldrich) at the concentration of 10 mM for 2 hours at 37°C before performing the assay. This approach stabilized the expression of CD23 on the cell surface of CLL cells for at least 24 hours (data not shown) and this allowed to perform the experiments. To adapt the 51\textsuperscript{Cr} release assay to smaller target cells like resting B cells and CLL cells, we optimized assay conditions by labelling resting B cells and CLL with 50\(\mu\text{Ci}\) of 51\textsuperscript{Cr} instead of 25\(\mu\text{Ci}\) for 1 hour instead of 45 minutes. We also increased the time of co-culture from 4 to 6 hours and reduced the final reaction volume from 200\(\mu\text{L}\) to 100\(\mu\text{L}\), thereby increasing the sensitivity of 51\textsuperscript{Cr} detection.
Short term co-culture experiments

The cytotoxic activity of NT and CAR+ T cells towards autologous CLL cells and normal donor derived B cells was also evaluated in a 24-hour co-culture assay using target cells labelled with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) (eBioscience, San Diego, CA, USA) as previously described. In these experiments, as described for the 51Cr release assay, CLL cells were pre-incubated with the metalloprotease-inhibitor Imidazole. We co-cultured NT and CAR+ T cells (9x10^5 cells/well) with 3x10^5 CFSE stained cells /well at a E:T Ratio 3:1 for 24 hours at 37°C. Cells were then collected, stained with CD3 to detect T lymphocytes, and analyzed by flow cytometry to quantify residual normal B or tumor cells as CFSE positive cells.

Long term co-culture experiments

To evaluate whether the soluble form of CD23 could affect the cytotoxic activity of CD23.CAR+ T lymphocytes, we co-cultured NT and CAR+ T cells (1x10^6 cells/well) with viable LCLs (E:T Ratio 1:1) in the presence or in the absence of serial dilutions of plasma enriched in soluble CD23 obtained from CLL patients. After 4 days of co-culture, cells were collected, stained with CD3 and CD19 monoclonal antibodies to detect T lymphocytes and tumor cells, respectively, and then analyzed by flow cytometry to quantify residual tumor cells.

Cell expansion

To evaluate the expansion of T lymphocytes in response to CD23+ cell lines (LCLs and MEC-1) and autologous CLL cells, NT and CD23.CAR+ T cells (1x10^6) obtained from HDs and CLL patients were stimulated once a week with allogeneic, γ-irradiated (30 rads) LCLs (E:T Ratio 1:1) or γ-irradiated (100 rads) MEC-1 (E:T Ratio 1:1) or non irradiated autologous CLL cells (E:T Ratio), pre-incubated with Imidazole as described above, without addition of exogenous cytokines. T lymphocytes were counted with Trypan blue (Sigma-Aldrich) to assess the fold increase rate at different time points.

Cytokine release

NT and CAR+ T lymphocytes (2x10^5 or 1x10^6) were co-cultured with γ-irradiated allogeneic LCLs or MEC-1 or non irradiated autologous CLL cells (E:T Ratio 1:1), and culture supernatants collected after 24 or 48 hours. IFN-γ, TNF-α and TNF-β were measured with a Flow Cytomix Assay (Bender Medsystem, Vienna, Austria), while IL-2 production was measured using a specific Enzyme-Linked Immunosorbent Assay (Peprotech, London, UK). Our data were normalized by subtracting the spontaneous release of cytokines from tumor cell lines or autologous CLL cells.
**Soluble CD23 antigen analysis**

Plasma samples from HDs and CLL patients were obtained by centrifugation (10 minutes at 2500 rpm) of fresh PB. The plasma fractions have been stored at -20°C until soluble CD23 quantification was performed. Soluble CD23 levels of HDs and CLL patients-derived plasma samples have been detected using a human CD23 ELISA kit (Bender MedSystem) according to manufacture’s instructions.

**In vivo studies**

Eight week-old Rag2\(^{-/-}\)γ\(_c\)\(^{-/-}\) female mice were injected subcutaneously (s.c.) in the left flank with 10x10\(^6\) MEC-1 cells in 0.1 ml saline. Ten days later, mice bearing subcutaneous tumor were adoptively transferred with a single intravenous (i.v.) infusion of either 10x10\(^6\) CD23.CAR\(^+\) T cells or NT cells obtained from HDs. Animals were then monitored twice a week for weight and tumor growth (measuring three perpendicular diameters), and sacrificed when the mean tumor volume reached a dimension of ≥1000 mm\(^3\), before reaching clinical signs and symptoms, in order to avoid unnecessary pain and discomfort according to standard ethical animal guidelines. Two separate experiments were performed with 3 mice per group each (manipulated and control/non manipulated T cells).

**Statistical analysis**

The data are reported as means and standard deviations (SD). The paired Student \(t\) test was used to determine the statistical significance of differences between samples. \(P\) values less than 0.05 were considered as statistically significant.
Results

Functional CD23.CAR can be expressed by activated T lymphocytes

We first evaluated whether CD23.CAR can be expressed by activated T lymphocytes and if these cells had cytotoxic activity against CD23+ target cells. T lymphocytes expressing CD19.CAR, previously largely validated, were used as positive control. Activated T lymphocytes obtained from the PB of HDs were transduced either with CD19.CAR or CD23.CAR retroviral supernatants. As shown in Fig. 1A, both CD19.CAR and CD23.CAR were efficiently expressed by T lymphocytes (average 58%, range 23-82%, and 58%, range 18-81%, n=10, respectively). Both CD4+ and CD8+ T lymphocytes were transduced with similar efficiency and the expression of the transgene was maintained in cells cultured for more than 2 weeks (data not shown). After transduction, both CD19.CAR+ and CD23.CAR+ cells expanded equally well in vitro in the presence of IL-2 (n=8)(Fig. 1B). Phenotype performed 3 days after gene transfer showed that T-cell lines were a mixture of CD4+ cells (average 64%, range 54-73% for CD23.CAR+ cells, and 68%, range 40-88% for CD19.CAR+ cells, n=6) and CD8+ cells (average 32%, range 24-40% for CD23.CAR+ cells, and 33%, range 21-44% for CD19.CAR+ cells, n=6). Less than 2% of CAR+ cells were CD3−CD56+ NK cells (Fig. 1C). To evaluate the cytotoxic activity of non transduced (NT) and CAR+ cells we used a 4 hours standard 51Cr release assay. Target cells were allogeneic EBV+ LCLs, which naturally express both CD23 and CD19 molecules and two CD23+ tumor cell line gene modified to express comparable levels of CD23 (CD23+ Jeko-1 and CD23+ BJAB ) (Fig. 2A). CD23.CAR+ T cells had a cytotoxic activity comparable to CD19.CAR+ T cells against allogeneic LCLs (average lysis 42%, range 27-54% at E:T ratio 40:1, and average lysis 47%, range 30-72% at E:T ratio 40:1, respectively, n=7, p≤0.005) (Fig. 2B). Less than 10% lysis was observed for NT lymphocytes (Fig. 2B). CD23.CAR+ cells also acquired specific cytotoxic activity against CD23+ Jeko-1 (n=6) (Fig. 2C) and CD23+ BJAB cells (n=6) (Fig. 2D), while these cells had negligible cytotoxic activity against wild type Jeko-1 (Fig. 2C) and BJAB (Fig. 2D) cells.

CD23.CAR+ T lymphocytes do not show cytotoxic activity against normal B lymphocytes

Since one potential concern associated with adoptive transfer of CD19.CAR+ T cells in patients with B-cell malignancies is the elimination of both leukemic and non malignant B lymphocytes, we investigated if CD23.CAR+ T cells could spare normal B lymphocytes as they express lower levels of the CD23 antigen. Whilst percentages of double positive CD19/CD23 cells were comparable in HDs and CLL patients at levels ≥ 90%, n=5 and n=7 respectively, (data not shown), as expected, the level of expression of CD23 antigen was higher in CD19+ CLL cells, when assessed as mean fluorescence intensity (MFI) (average MFI 507, range 356-704, n=7 for CD19+ CLL cells and average MFI 183, range 97-273, n=5 for normal CD19+ B lymphocytes, p≤0.005) (Fig. 3A). We then analyzed the cytotoxic effects of either CD23.CAR+ or CD19.CAR+ T lymphocytes against normal B lymphocytes. As shown in Fig. 3B, lysis of normal B lymphocytes by CD23.CAR+ cells (average lysis 13%, range 7-17%, at an E:T ratio 40:1, n=3) was significantly lower as compared to lysis by CD19.CAR+ T cells (average lysis 77%, range 57-100%, at an
E:T ratio 40:1, n=3, p≤0.05) and similar to that of NT cells, that was less than 15%. We also evaluated the individual cytotoxic effects of CD23.CAR+ or CD19.CAR+ T lymphocytes against normal B lymphocytes in 24-hour co-culture experiments using CFSE labelled target cells. As shown in Fig 3C, the 24-hour co-culture experiments confirmed that CD23.CAR+ T CLL derived T cells had only a marginal cytotoxic effect against normal B lymphocytes compared to CD19.CAR+ cells.

CD23.CAR+ T cells generated from CLL patients have cytotoxic activity against CLL cells

As described for T-cell lines generated from HDs, we efficiently generated CD23.CAR+ T lymphocytes from samples collected from CD23+ CLL patients. The expression of CD23.CAR and CD19.CAR was 71% (range, 45-90%) and 68% (range, 47-92%, n=10), respectively. Phenotypic profile and expansion rate of these T-cell lines (data not shown) were similar to those observed for T-cell lines generated from HDs (Fig 1A and 1B). Moreover, since Tregs may be abundant in PB of CLL patients,8,30,31, we measured the presence of these cells by phenotypic analysis before and 14 days after CAR gene transfer. The percentages of CD4+CD25+FoxP3+ cells before and 14 days after gene transfer were similar (1.2%, range 0.9-1.6% and 0.8%, range 0.2-1.9%, n=5 respectively).

As illustrated in Fig. 4A, CD23.CAR+ T cells showed efficient cytotoxic activity against LCLs, as assessed by $^{51}$Cr release assay (average lysis 54%, range 24-70%, at E:T ratio 40:1, n=5, p≤0.05 when compared to NT lymphocytes), which was comparable to the cytotoxic effects of CD19.CAR+ T cells (average lysis 63%, range 40-75%, at E:T Ratio 40:1, n=5, p≤0.005 when compared to NT lymphocytes). Importantly, the anti-tumor effect of CD23.CAR+ T cells was not inhibited by the addition of human plasma enriched in soluble CD23 (2000 U/ml), as assessed in co-culture experiments (n=3) (Fig. 4B). The average percentage of residual CD19+CD23+ tumor cells in presence of CD23.CAR+ T cells was 15% (range 13-17%) in the absence of plasma, 17% (range 13-20%) in the presence of 25% of plasma and 12% (range 7-18%) in the presence of 50% of plasma (n=3 CLL donors). By contrast, residual CD19+CD23+ tumor cells were 44% (range 31-63%), 51% (range 39-68%) and 47% (range 30-66%), respectively, when control NT lymphocytes were used regardless the addition of soluble CD23 (n=3). We then evaluated the capacity of CAR-redirected T cells to specifically target autologous and allogeneic CD23+ CLL cells. CD23.CAR+ T cells efficiently lysed both autologous CD23+ leukemic cells (average lysis 58%, range 26-84%, at E:T ratio 20:1, n=3) (Fig. 4C) and allogeneic CD23+ leukemic cells (average lysis 51%, range 13-76%, at E:T ratio 20:1, n=3) using a 6 hour $^{51}$Chromium release assay (Fig. 4E). Co-culture experiments using CFSE labelled target cells also showed substantial elimination of autologous CD23+ leukemic cells within 24 hours (average lysis 66%, range 30-68%, at E:T ratio 3:1, n=4) (Fig. 4D). These effects were comparable to those obtained using CD19.CAR+ cells using autologous CLL cells in a $^{51}$Cr release assay (average lysis of autologous CLL cells, 72%, range 18-100%, at E:T ratio 20:1, n=3), in a 24 hour co-culture CFSE assay (average lysis of autologous CLL cells, 78%, range 69-88%, at E:T ratio 3:1, n=2), and using allogeneic CLL cells in a $^{51}$Cr release assay (average lysis of allogeneic CLL, 64%, range 18-100%, n=3).
CD23.CAR⁺ T lymphocytes generated from both HDs and CLL patients produce immunostimulatory cytokines in response to CD23⁺ tumor cells

NT and CD23.CAR⁺ T cells generated from either HDs or CLL patients were co-cultured with γ-irradiated LCLs (E:T Ratio 1:1). As shown in Figs. 5A and B, CD23.CAR⁺ T cells derived from HDs secreted Tumor Necrosis Factor-β (TNF-β) (497 pg/ml, range 460-529 pg/ml, n=3, p≤0.005) in contrast to NT lymphocytes. In addition, transduced T cells secreted >25 fold more Tumor Necrosis Factor-α (TNF-α) (426 pg/ml, range 325-483 pg/ml vs. 16 pg/ml, range 10-20 pg/ml, n=3, p≤0.05) and about 7 fold more Interferon-γ (IFN-γ) (3878 pg/ml, range 3393-4539 pg/ml vs. 561 pg/ml, range 184-928 pg/ml, n=3, p≤0.005) as compared to NT cells. Similarly, CD23.CAR⁺ T cells generated from CLL patients secreted TNF-β (1445 pg/ml, range 610-1990 pg/ml, n=4, p≤0.05) that was not produced by NT cells. In addition, transduced T cells secreted 4 fold more IFN-γ (5023 pg/ml, range 2860-9235 pg/ml vs. 1340 pg/ml, range 265-3347 pg/ml, n=4, p≤0.05) and 20 fold more TNF-α (245 pg/ml, range 77-435 pg/ml vs. 12 pg/ml, range 0-25 pg/ml, n=4, p≤0.05), as compared to NT lymphocytes. No major differences in cytokine secretion were detected between CD23.CAR⁺ and CD19.CAR⁺ T cells (data not shown). As illustrated in Supplementary Fig. 1, CD23.CAR⁺ T cells also produced inflammatory cytokines against autologous CLL cells and the MEC-1 cell line.

CD23.CAR⁺ T cells produce IL-2 and proliferate in response to CD23⁺ target cells

Since our novel CAR includes the CD28 co-stimulatory endodomain, we evaluated whether CD23.CAR⁺ T cells released IL-2 and proliferated in response to CD23⁺ target cells (LCLs and MEC-1) and autologous CLL cells as described for other CARs incorporating the same co-stimulatory endodomain.17,25,30 As shown in Fig. 6A, CD23.CAR⁺ T cells generated from HDs released IL-2 after stimulation with γ-irradiated allogeneic LCLs (E:T Ratio 1:1) (average 14229 pg/ml, range 9262-19539 pg/ml, n=4), while no IL-2 was produced by NT lymphocytes (0 pg/ml, p≤0.05). Similarly, CD23.CAR⁺ T cells generated from CLL samples produced IL-2 in response to CD23⁺ target cells (LCLs) (average 2681 pg/ml, range 2411-2833 pg/ml, n=3) (Fig. 6B). As shown in Figs. 6C and D, CD23.CAR⁺ T cells proliferated in response to the CD23⁺ LCLs without the addition of exogenous IL-2. After 4 weeks of culture CD23.CAR⁺ T cells showed an average fold increase of 38 (range 20-65, n=3) and 18 (range 14-24, n=4), when generated from HDs and CLL samples, respectively. In contrast, control NT lymphocytes did not proliferate in response to CD23⁺ targets (average fold increase less than 2 for both HDs and CLL samples). As shown in Supplementary Fig. 1, CD23.CAR⁺ modified T cells also proliferated in response to either MEC-1 cell line or autologous CLL cells.

CD23.CAR⁺ T cells provide in vivo antitumor effect in the Rag2⁻/⁻γc⁻/⁻ xenograft model of CLL

To investigate the antitumor activity of CD23.CAR⁺ T lymphocytes in vivo, MEC-1 cells were injected s.c. into the left flank of Rag2⁻/⁻γc⁻/⁻ female mice. Ten days later, when tumors had reached a mean volume of 44 ±8.3 mm³, either CD23.CAR⁺ or NT T lymphocytes were injected i.v. in the animals that were then
monitored for tumor growth. The follow-up was interrupted at day 33 when the mean tumor volume reached 1056 mm$^3$ in the group with NT cells. Adoptive cellular transfer did not modify the animal weight as compared to control (NT cell-injected animals, data not shown) but, interestingly, already at day 12, mice receiving CD23.CAR$^+$ T cells showed a significant decrease of the MEC-1 tumor growth when compared with control mice as reported in Fig. 7 panel A (CD23.CAR$^+$ T cells vs NT T cells; day 12 ($p \leq 0.05$); day 14($p \leq 0.05$); day 21($p \leq 0.05$); day 28 ($p \leq 0.05$); day 31 ($p \leq 0.005$); day 33 ($p \leq 0.05$); n=3. These in vivo results were confirmed when repeated in a second independent experiment where MEC-1 transplanted Rag2$^{-/-}$γc$^{-/-}$ mice were injected either with CD23.CAR$^+$ T cells or NT T cells obtained from a different donor as shown in Fig. 7 panel B (CD23.CAR$^+$ T cells vs NT T cells; day 31 ($p \leq 0.05$); day 33 ($p \leq 0.05$); n=3).
Discussion

Due to its chronic clinical course and the need to reduce treatment-related toxicity, CLL is suited to immunotherapy-based approaches. Adoptive transfer of T lymphocytes genetically modified to express CARs conveniently combines both antibody-mediated specificity and T-cell effector functions. Because the ideal tumor associated antigen should be expressed by tumor cells, but virtually absent in normal tissues, we have taken advantage of the selective higher expression of CD23 by CLL cells to target malignant cells, whilst sparing normal B cells. We have cloned a fully humanized CAR specific for the CD23 antigen, which is highly expressed by CLL cells, and showed that T lymphocytes carrying this receptor efficiently eliminated CD23+ tumor cell lines and primary CLL cells, whilst sparing normal B cells. Being able to preserve the normal B cell compartment in CLL patients represents an important improvement, as it would preserve their humoral immunity, already often impaired.

When targeting CD23, our primary concern was to show lack of activity toward normal B cells. CD23 has been implicated in normal B-cell proliferation and survival and although CD23-deficient mice have normal B-cell development indicating that the elimination of CD23+ cells may not compromise the normal B-cell compartment, we carefully evaluated the effects of CD23.CAR-specific T cells on normal B lymphocytes. These cells can indeed express CD23, especially as IgM- and IgD-positive B cells in the mantle zone of the secondary follicle, and as illustrated in our experiments, they can circulate in the peripheral blood of healthy individuals. However, their expression of CD23 is low, and, accordingly, we did not detect significant CD23.CAR-mediated cytolytic activity against these cells. Since normal B lymphocytes are not intrinsically resistant to the cytotoxic effects of CAR-redirected T cells, as illustrated by their elimination by CD19.CAR-specific T cells, we suggest that the lack of cytoxicity by CD23.CAR+ T cells on these cells is likely the result of both the low absolute number of CD23 molecules per cells (as indicated by the low MFI of the membrane bound CD23) and/or a suboptimal affinity of the single chain cloned from the CD23 antibody, even if the native antibody may have high affinity. Indeed, the cross linking of CAR molecules, required for the dimerization of the ζ chains and activation of CAR-redirect T cells, is influenced by both the antigen density on target cells and the affinity of the single chain antibody. As a consequence, if T cells express a CAR with relatively low affinity, successful activation of T lymphocytes to eliminate target cells can only occur when the antigen is highly expressed as it occurs on the high-proliferating subset of CD23brigh CLL cells, but not on the CD23dim expressing normal B cells.

CD23 seems then a good target for CLL as it is highly expressed on leukemic cells, contributes in sustaining their viability and appears essential for maintaining their aggressive neoplastic phenotype. CD23 can be cleaved from CLL cells, with high levels of the soluble form correlating with disease activity and poor clinical outcome. Although soluble antigens may reduce the bio-availability of monoclonal antibodies and impair their anti-tumor effects, likely accounting for the limited efficacy of the CD23-specific Lumiliximab as single agent, as compared to a combined treatment, we showed that CD23.CAR+ T lymphocytes do not suffer from these competitive effects. This was however not a surprising phenomenon,
since similar results were previously observed for CARs targeting other antigens also present in a soluble form such as GD2, CD30, CEA and the light immunoglobulin chains. Of note, the observed lack of cytolitic activity against normal B lymphocytes was not due to a rapid loss of membrane bound CD23 by normal B cells in culture as cytofluorimetric analysis shows stable CD23 expression by normal B lymphocytes for at least 24 hours in culture even in the absence of metalloprotease inhibitors (data not shown).

Previous studies have shown that CD23 can be processed and presented in the context of the MHC molecules and that HLA-A2-restricted cytotoxic T lymphocytes (CTLs) specific for CD23-derived peptides can be elicited from the peripheral blood of CLL patients and recognize CD23+ CLL cells. Although the generation of CTLs targeting tumor associated antigens (TAAs) using the native αβ T-cell receptor is a feasible approach, the large scale production of these cells remains challenging. The lack or limited availability of professional antigen presenting cells for ex vivo T-cell expansion, the paucity of TAA-specific CTL precursors and their anergy or hypo-responsiveness often observed in cancer patients, including CLL patients, represent major limitations to the success of this strategy. By contrast, a CAR-based approach allows the rapid generation of large number of tumor-specific T cells that function in a non MHC-restricted fashion, and thus applicable to all CLL patients.

Two more advantages can be envisioned for CAR-based strategies particularly when targeting CLL. First, this approach overcomes the anergy often observed for CLL-derived T cells, as after CAR gene transfer, T-cell lines generated from CLL patients released immunostimulatory cytokines such as IFN-γ, TNF-α and TNF-β in response to CD23+ cell targets. Second, the lack of co-stimulatory molecules by CLL cells will not impair effective T-cell activation and persistence. Indeed, the incorporation of the CD28 co-stimulatory endodomain within the CD23.CAR construct, allowed production of IL-2 that sustained their expansion in response to primary tumors suggesting enhanced activity in vivo after adoptive transfer. The CD28 endodomain should also help in sustaining cytotoxic activity and proliferative response of CAR redirected T cells in the presence of regulatory T cells, which are particularly abundant in CLL patients.

The therapeutic potential of our CD23.CAR was finally validated using a recently published murine xenograft model of human CLL. This model, based on either s.c or i.v injection of the CLL derived cell-line MEC-1 in an immunodeficient Rag2γc-/- mouse, resembles the aggressive form of human CLL in terms of disease development, tissue distribution and organ involvement, and, because these mice lack B, T and NK cells, it reveals therapeutic effect exclusively mediated by the adoptively transferred T cells. Our in vivo experiments showed that CD23.CAR+T cells were able to significantly control growth of CD23+ tumor cells for more than 3 weeks after one single intravenous dose of T cells, without any systemic addition of exogenous cytokines.

In conclusion, CD23.CAR-redirected T cells provide cytotoxic activity against CD23+ CLL cells in vitro and in vivo, while sparing normal B lymphocytes, as compared to other available CARs targeting pan-B-cell antigen such as CD19. The selection of this antigen offers advantages as the most aggressive fraction of CLL cells, present within the proliferation centers and expressing higher levels of the CD23 antigen, will be
efficiently targeted. Ultimately, clinical studies are required to discover if targeting the CD23 molecule offers a significant benefit, rather than targeting a pan–B-cell antigen.
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Author contributions

GMPGA, VM, PG, GD and EB designed the research, critically analyzed the data and wrote the paper. GMPGA, VH, BS, IP, ST and VA performed the in vitro experiments. GMPGA and MTSB performed the in vivo animal studies. MP and AB contributed in the analysis of the data and in the writing of the paper and MP in setting up the animal studies and evaluation of in vivo response.

Conflict of Interest Disclosure

The authors declare no competing financial interests.
References


Figure legends

**Fig. 1. Activated T lymphocytes can efficiently express either CD23.CAR or CD19.CAR.**

(A) CAR expression in CD19.CAR and CD23.CAR-redirected T lymphocytes for one representative experiment out of 10. Control NT T cells are also included.

(B) Median fold expansion of NT (white diamonds), CD19.CAR+ (striped squares) and CD23.CAR+ (black triangles) T lymphocytes generated from HDs, and cultured for 9 days with IL-2 upon retroviral-mediated gene transfer. Mean and SDs are shown for 8 different T-cell lines.

(C) Phenotype of NT (white bars), CD19.CAR+ (striped bars) and CD23.CAR+ (black bars) T lymphocytes generated from HDs and stained 3 days upon retroviral gene transfer. Mean and SDs are shown for 6 different T-cell lines.

**Fig. 2. CD23.CAR+ and CD19.CAR+ T lymphocytes have equal cytotoxic activity against CD19+CD23+ tumor cells.**

(A) Expression of CD23 antigen on target cells used in the cytotoxicity assay.

(B) Cytotoxic activity of CD19.CAR+ and CD23.CAR+ T lymphocytes obtained from HDs against CD23+ LCL targets. Cytotoxic activity was evaluated in a standard 4-hour Cr51 release assay, and results are shown at the E:T ratios of 40:1, 20:1, 10:1, 5:1. Data represent the mean ± SD of 7 T-cell lines. ** Indicates p≤0.005 when comparing NT and CAR+ T cells. The difference between the cytotoxic activity of CD19.CAR+ and CD23.CAR+ T lymphocytes is not statistical significant at any given E:T ratio.

(C) Cytotoxic activity of CD23.CAR+ T-lymphocytes generated from HDs against the wild type Jeko-1 cell line or CD23+ cells. Cytotoxic activity was evaluated in a Cr51 release assay, and results are shown at the E:T ratios of 40:1, 20:1, 10:1 and 5:1. Data represent the mean ± SD of 6 T-cell lines. ** Indicates a p≤0.005 when comparing NT and CAR+ T cells. (D) Cytotoxic activity of CD23.CAR+ T-lymphocytes generated from HDs against the wild type BJAB cell line or CD23+ cells. Cytotoxic activity was evaluated in a Cr51 release assay, and results are shown at the E:T ratios of 40:1, 20:1, 10:1 and 5:1. Data represent the mean ± SD of 6 T-cell lines. ** Indicates a p≤0.005 when comparing NT and CAR+ T cells.

**Fig. 3. CD23.CAR+ T lymphocytes lack cytotoxic activity against normal B lymphocytes.**

(A) The mean fluorescence intensity (MFI) of CD23 antigen on B cells isolated from the PB of HDs and CLL patients is shown. Percentages of double positive CD19/CD23 cells were comparable in HDs and CLL patients at levels ≥ 90% (data not shown), as expected. ** Indicates a p≤0.005 when comparing HDs and CLL derived B cells.

(B) Cytotoxic activity of control NT (white bar), CD19.CAR+ (striped bars) and CD23.CAR+ (black bars) T lymphocytes obtained from HDs against purified CD20+ B cells obtained from the PB of HDs. Cytotoxic activity was evaluated in a Cr51 release assay, and results are shown at the E:T ratios of 40:1, 20:1, 10:1 and
5:1. Data represent the mean ± SD of 3 different T-cell lines. * Indicates a p≤0.05 when comparing CAR⁺ to NT lymphocytes.

(C) Cytotoxic activity of control NT, CD19.CAR⁻ and CD23.CAR⁺ T lymphocytes obtained from CLL patients against purified CD20⁺ B cells labelled with CFSE. Cytotoxic activity was evaluated in a 24 hours co-culture experiment, and results are shown at the E:T ratio of 3:1. The experiment is representative of 3 different experiments.

Fig. 4. Activated T lymphocytes generated from CLL patients are efficiently transduced with CAR-encoding retroviral vectors and have cytotoxic activity against CD23⁺ tumor cells.

(A) Cytotoxic activity of NT (white bar), CD19.CAR⁺ (striped bars) and CD23.CAR⁺ T lymphocytes (black bars) generated from CLL donors and tested against allogeneic LCLs. Data represent the mean ± SD of 5 T-cell lines. * and ** indicate a p≤0.05 and a p≤0.005, respectively when comparing CAR⁺ to NT lymphocytes.

(B) Dot plot analysis of the co-culture experiments in which CD23.CAR⁺ T cells derived from CLL patients were co-cultured with allogeneic LCLs in the absence or in the presence of different percentages of plasma enriched in soluble CD23. One representative experiment is shown. The numbers represent the percentage of residual CD19⁺ LCLs cells (tumor cell line) enumerated by FACS analysis after 4 days of co-culture. The experiment is representative of 3 different experiments.

(C) Cytotoxic activity of NT (white bar), CD19.CAR⁺ (striped bars) and CD23.CAR⁺ (black bars) T lymphocytes generated from CLL patients and tested against autologous CD23⁺ CLL cells. Data represent mean ± SD of 3 T-cell lines. * Indicates a p≤0.05 , when comparing transduced to NT lymphocytes.

(D) Cytotoxic activity of NT (white bar), CD19.CAR⁺ (striped bars) and CD23.CAR⁺ (black bars) T lymphocytes generated from CLL patients and tested against autologous CD23⁺ CLL cells using CFSE staining and co-cultured for 24 hours. Data represent mean ± SD of 4 T-cell lines. * Indicates a p≤0.05 , when comparing CAR⁺ transduced to NT lymphocytes.

(E) Cytotoxic activity of NT (white bar), CD19.CAR⁺ (striped bars) and CD23.CAR⁺ (black bars) T lymphocytes generated from CLL patients and tested against allogeneic CD23⁺ CLL cells. Data represent mean ± SD of 3 T-cell lines. * Indicates a p≤0.05 , when comparing transduced to NT lymphocytes.

Fig. 5. CD23.CAR⁺ T lymphocytes generated from HDs and CLL patients produce inflammatory cytokines in response to CD23⁺ tumor cells.

CD23.CAR⁺ T cells generated from HDs (A) and CLL patients (B) produced higher levels of TNF-β, TNF-α and IFN-γ in response to allogeneic LCLs as compared to NT cells. Data represent mean ± SD of 3 and 4 T-cell lines generated from HDs and CLL patients, respectively. * and ** Indicates a p≤0.05 and a p≤0.005, when comparing CAR⁺ transduced to NT lymphocytes.
Fig. 6. CD23.CAR⁺ T lymphocytes generated from HDs and CLL patients produce IL-2 and proliferate in response to CD23⁺ tumor cells.

(A) and (B) CD23.CAR⁺ T cells generated from HDs and CLL patients produced IL-2 when stimulated with allogeneic LCLs (1:1 E:T ratio) as compared to NT lymphocytes. Data represent mean ± SD of 4 and 3 T-cell lines generated from HDs and CLL patients, respectively. * Indicates a p ≤ 0.05, when comparing transduced to NT lymphocytes.

(C) and (D) Expansion of CD23.CAR⁺ T cells derived from HDs and CLL patients in response to allogeneic LCLs as compared to NT lymphocytes. Cells were stimulated once a week without the addition of exogenous cytokines. Data represent mean ± SD of 3 and 4 T-cell lines generated from HDs and CLL patients, respectively. * Indicates a p ≤ 0.05, when comparing transduced to NT lymphocytes.

Fig. 7. CD23.CAR⁺ T cells control tumor growth in a CLL xenograft model.

(A) and (B) 10x10⁶ MEC-1 tumor cells were inoculated subcutaneously in the left flank of Rag2⁻/⁻γc⁻/⁻ male mice. To test the therapeutic efficacy of CD23.CAR⁺ T cells mice bearing an established tumor were inoculated i.v. with 10x10⁶ NT or CD23.CAR⁺ T cells derived from different donors for each experiment. Tumor growth was then monitored as tumor diameter/day. Data represent the mean ± SD of 3 mice for each panel presented. * and ** indicates a p ≤ 0.05 or p < 0.005, respectively when comparing CD23.CAR⁺ to NT lymphocytes.
Figure 2

A

B

C

D
Figure 4

A

B

C

D

E
Figure 5

A

[Bar charts showing cytokine levels in HDs (Healthy Donors) and TNF-β/α and IFN-γ levels with statistical significance marked by asterisks (**) and (*)].

B

[Bar charts showing cytokine levels in CLL patients (Chronic Lymphocytic Leukemia) and TNF-β/α and IFN-γ levels with statistical significance marked by asterisks (*)].
Figure 7

A

B

Tumor Volume (mm$^3$)

Time (Days)

- CD33.CAR
- NT

Tumor Volume (mm$^3$)

Time (Days)

- CD33.CAR
- NT
In vitro and in vivo model of a novel immunotherapy approach for chronic lymphocytic leukemia by anti-CD23 chimeric antigen receptor

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