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Allogeneic cellular immunotherapy for chronic B-cell leukemia

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Chapter 4

Primary allogeneic T-cell responses against mantle cell lymphoma antigen presenting cells for adoptive immunotherapy after stem cell transplantation

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Abstract

Purpose: In patients, treated with allogeneic stem cell transplantation (SCT) for advanced mantle cell lymphoma (MCL), complete sustained remissions have been observed illustrating susceptibility of MCL cells to a graft-versus-lymphoma (GvL) effect. To potentiate this GvL effect adoptive transfer of *in vitro*-selected MCL-specific cytotoxic T cells (CTLs) can be an attractive approach. The lack of expression of costimulatory molecules on MCL cells hampers the generation of MCL-reactive T cell-responses. The purpose of this study was to modify MCL cells into antigen-presenting cells (APC) and to use these MCL-APC to induce allogeneic MCL-reactive T-cell responses.

Experimental Design: Interleukin (IL)-4, IL-10, CpG, and CD40 activation were tested for their capacity to upregulate costimulatory molecules on MCL cells. Primary MCL cells or the modified MCL-APC were then used to evaluate the induction of MCL-reactive T-cell responses in HLA matched donors.

Results: Ligation of CD40 on MCL cells was essential to upregulate costimulatory molecules and to induce production of high amounts of IL-12. In contrast to primary MCL cells, MCL-APC cells as stimulators were capable of inducing cytotoxic CD8⁺ T-cell (CTL) lines from HLA class I-matched donors. High numbers of CTL clones could be generated capable of efficiently killing the primary MCL cells and MCL-APC but not donor-specific targets.

Conclusion: These results demonstrate the feasibility to generate primary allogeneic T-cell responses against MCL-APC, and may provide new immunotherapeutic tools to further exploit the GvL effect following allogeneic SCT in patients with MCL.

Introduction

Mantle cell lymphoma (MCL) is a non-Hodgkin lymphoma (NHL), characterized by the t(11;14)(q13;q32) translocation involving the PRAD-1/bcl 1 gene resulting in overexpression of cyclin D₁¹. Patients with MCL frequently present with advanced-stage disease and have a median survival of only 3 to 4 years². Although in general MCL is initially responsive to conventional chemotherapy or to high-dose chemoradiotherapy followed by autologous stem cell transplantation (SCT), persistent remissions are usually not achieved reflected in the absence of a plateau phase in the survival curves^{3,4}. The addition of rituximab to autologous SCT as an *in vivo* purging agent or as maintenance therapy appears promising, but has not yet definitively shown to result in a survival benefit⁵⁻⁷.

Allogeneic SCT is currently being explored as a treatment modality in patients with advanced or relapsed chemosensitive MCL. Complete sustained remissions have been observed after allogeneic SCT illustrating susceptibility of MCL cells to a graft-versus-lymphoma (GvL) effect^{5,8}. High treatment related mortality (TRM) has hampered large scale application of allogeneic SCT in this extensively pretreated older patient population. Allogeneic SCT following reduced-intensity conditioning (RIC) has been shown to be feasible and resulted in a lower TRM, allowing the application of non-selected donor lymphocyte infusion (DLI)⁹⁻¹². However, acute and chronic graft-versus-host disease (GvHD) caused by alloreactive T cells present in the graft and/or in the DLI and persistence or recurrence of MCL after allogeneic SCT has limited favourable long-term outcomes¹³. Therefore, both the specificity of the immune reactivity in the context of allogeneic SCT, as well as the magnitude of the immune response specific for MCL cells have to be improved. The use of T-cell depleted grafts avoiding the risk of GvHD, followed by *in vitro* selected alloreactive T cells with specificity for the MCL cells or for hematopoiesis-restricted minor histocompatibility antigens (mHag) could be an attractive approach, and may induce complete remissions with only minor graft-versus-host disease (GvHD)¹⁴.

Malignant B cells inefficiently induce allogeneic T-cell responses despite their strong expression of HLA class I and class II due to inadequate expression of costimulatory and adhesion molecules¹⁵⁻¹⁷. However, both normal and malignant B cells highly express CD40 molecules. Ligation of these receptors induces expression of the costimulatory molecules CD80 and CD86, adhesion molecules such as CD54 and CD58, and upregulates cytokine production¹⁶⁻²¹. Stimulation of normal B cells can also be initiated by activating toll-like receptor (TLR) 9. TLR 9, expressed on normal and various neoplastic B cells²²⁻²⁵, detects CpG motifs within bacterial DNA and induces upregulation of costimulatory molecules on these cells. Synthetic CpG oligodeoxynucleotide (ODN) 2006 mimic microbial DNA and has the ability to activate normal and malignant B cells²⁶. Transformation of B cell malignancies, including B-cell chronic lymphocytic leukemia, follicular lymphoma and hairy cell leukemia, into antigen-presenting cells (APC) has been studied previously^{15,17,20,21,27-29}, but the modification of MCL cells into APC and the generation of MCL-reactive T-cell responses have not been reported.

In this study, we hypothesized that by transforming MCL cells into professional APC and using these MCL-APC to stimulate HLA-matched donor T cells, the generation of MCL-reactive cytotoxic T lymphocytes (CTL) lines will be possible. We first examined the stimulatory capacity of cytokines, including the proinflammatory cytokines TNF- α and interferon- α , the B-cell activating cytokine interleukin (IL)-4³⁰, and the MCL-stimulating cytokine IL-10³¹ and the additional triggering of the TLR9 by the synthetic agonist CpG ODN 2006, or triggering CD40 with CD40 ligand transfected fibroblasts (tCD40L) to upregulate costimulatory molecules on MCL cells, and to induce the production of IL-12 by these cells. Using CD40-activated MCL cells as stimulator cells, we could demonstrate that MCL-reactive mHag-specific CTL lines and clones from HLA class I-matched donors could be generated. These anti-MCL T-cell responses may be used to treat patients, suffering from relapse of MCL after allogeneic SCT.

Material and methods

Cell samples

After informed consent peripheral blood samples were obtained from 6 patients with MCL in the leukemic phase. The diagnosis of MCL was confirmed by detection of cyclin D₁ overexpression using cytohistochemical staining and verification of the t(11;14) translocation by karyotype or interphase fluorescence in situ hybridization (FISH)³². The patient characteristics are shown in table 1.

Table 1. Patient characteristics.

Patient	sex/age/stage	phenotype	cyclin D1	t (11;14)
MCL 1	M / 68 / IV	CD5+CD23-	+	+
MCL 2	M / 44 / IV	CD5+CD23-	+	+
MCL 3	M / 56 / IV	CD5+CD23-	+	+
MCL 4	M / 88 / IV	CD5+CD23±	+	+
MCL 5	V / 52 / IV	CD5+CD23-	+	+
MCL 6	M / 64 / IV	CD5+CD23-	+	+

M, male; F, female. Cyclin D1 overexpression was detected using cytohistochemical staining. The t(11;14) translocation was verified by karyotype or FISH analysis.

Mononuclear cells (MNC) were isolated from peripheral blood samples by Ficoll density separation, and cryopreserved. The percentage of CD5+/CD19+ MCL cells in the MNC fraction was always higher than 90%. HLA-A, -B, and -C typing of patients was performed by standard serology methods, and HLA-DR and -DQ typing was done by DNA analysis using sequence specific primers. To induce T-cell responses, MNC from a fully HLA class I-matched URD and an HLA-A- and HLA-B-matched unrelated donor (URD) were tested against MCL 3 and MCL 4, respectively (table 2).

Table 2. HLA type of patients and unrelated donors (URD).

Donor/patient	HLA class I						HLA class II			
	A2	A33	B15	B58	Cw3		DR13*	DR15	DQ6*	
MCL 3	A2	A33	B15	B58	Cw3		DR13*	DR15	DQ6*	
URD	A2	A33	B15	B58	Cw3		DR17	DR15	DQ1	DQ3
MCL 4	A1	A11	B37	B44	Cw6	Cw16*	DR7*	DR15	DQ2*	DQ6*
URD	A1	A11	B37	B44	Cw6	Cw5	DR3	DR4	DQ1	DQ7

HLA-A, -B, and -C typing was performed by standard serology methods. HLA-DR and -DQ typing was done by DNA analysis using sequence specific primers. *donor-patient disparate alleles

Transformation of MCL cells into APCs

MCL cells were cultured in medium consisting of IMDM (BioWhittaker, Verviers, Belgium) with 10 % human serum in 6-well plates (Costar, Cambridge, MA, USA) at a concentration of 10^6 cells/mL in a total volume of 5 mL per well. MCL cells were cultured in the presence or absence of the cytokines IL-4 (500 U/mL, Schering-Plough, Amsterdam, the Netherlands), IL-10 (10 ng/mL, Sanquin, Amsterdam, The Netherlands), TNF- α (10 ng/mL, Boehringer Ingelheim, Ingelheim am Rhein, Germany) or interferon- α (IFN- α 1000 U/mL, Hoffmann-La Roche, Basel, Switzerland), or in the presence or absence of the cytokine combinations IL-4 and IL-10. For triggering TLR 9, MCL cells were incubated with its ligand synthetic CpG ODN 2006 (CpG; 5' TCGTCGTTTTGTCGTTTTGTCGTT-3', 10 μ g/mL; Eurogentec, Seraing, Belgium) in the presence or absence of the cytokine combination IL-4 and IL-10. Finally, to further enhance upregulation of costimulatory molecules MCL cells were cocultured on Itk murine fibroblast cells transfected with the human CD40-ligand³³ (tCD40L; kindly provided by Dr. C. van Kooten, Department of Nephrology, Leiden University Medical Center). The fibroblasts were irradiated (70 Gy), and seeded as a feeder layer at a concentration of 5×10^5 cells/well in 6-well plates (Costar). MCL cells were added at a concentration of 5×10^6 cells/well in presence or absence of the cytokines, cytokine combinations and/or immunostimulators as described above. After 1, 2, 4 or 6 days of culture, MCL cells were harvested and washed. Cytospin preparations were made on day 4 for morphological assessment by May-Grunwald-Giemsa (MGG) staining. The number of viable cells was counted using eosin death cell exclusion, and the cells were analyzed by flow cytometry.

Immunophenotyping and cytokine analysis

To perform immunophenotyping, a panel of mouse MoAbs conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) was used. These MoAbs included FITC-conjugated IgG1 antibodies specific for CD3, CD4, CD5, CD40, CD54 or HLA-DR, and FITC-conjugated IgG2a antibodies specific for CD58. PE-conjugated IgG1 antibodies specific for CD14, CD19, CD23, CD56 or CD80, PE-conjugated IgG2a antibodies specific for CD8, and PE-conjugated IgG2b antibodies specific for CD83 and CD86 were used. Appropriate isotype controls (IgG1, IgG2a and IgG2b) were used. All MoAbs were purchased from Coulter (Coulter Corporation, Miami, Florida, USA) except anti-CD40 (Serotec, Oxford, England). Cytoplasmatic TLR9 was measured using PE-conjugated IgG2a antibodies

(eBioscience, Anaheim, CA, USA). A quantity of 25 μL of each MoAb in the appropriate dilution was added to 10^5 cells in 50 μL PBS containing 0.8 g/L albumin (Sanquin). After 30 minutes of incubation at 4°C, the cells were washed twice, and analyzed on a Coulter FACScan (Coulter). Results were analyzed using the Coulter Expo 32 software (Coulter). The relative expression of surface antigens is described as the mean fluorescence intensity ratio (MFIR). This value is calculated by dividing the MFI of cells stained with fluorochrome-conjugated antigen-specific MoAb by the MFI of cells stained with fluorochrome-conjugated isotype-control MoAb. If the percentage of positive events was more than 10% the leukemic sample was considered positive for that surface marker, and then the MFIR was calculated. Cell-free supernatants were harvested after 96 hours of culturing the MCL cells with or without cytokines, with or without CpG, and in the presence or absence of irradiated tCD40L cells. Cytokine measurements were performed using commercial IL-10 (Sanquin), IL-12 p40/70 (U-CyTech, Utrecht, the Netherlands) and IL-12 p70 (U-Cy Tech) ELISA kits according to the manufacturer's instructions.

Mixed lymphocyte reaction

A modification of the allogeneic mixed lymphocyte reaction (MLR)³⁴ was used to analyze the functional significance of IL-10 and IL-12 to the APC capacity of the MCL cells. Freshly thawed or CD40-activated MCL cells from patients 3, 4 and 5 were used as stimulator cells. MNC from a healthy donor was used as responder cells. In a 96-well flat-bottom culture plates (Costar) responder cells at concentration of 1.5×10^4 were stimulated with 1×10^5 irradiated (15Gy) stimulator cells in the presence of IL-10 (10ng/mL), IL-12 (5 U/mL) or without cytokines. After 4 days of culture in IMDM with 10% human serum, 1 μCi of [³H]thymidine (Amersham, Roosendaal, The Netherlands) was added and after additional 18 hours of culture [³H]thymidine incorporation was measured. As a control, [³H]thymidine incorporation in wells containing responder or stimulator cells only was used. All counts were corrected by subtracting the mean stimulator control counts (18-fold) and the mean of responder control counts (6-fold). A Stimulation Index (SI) was calculated by dividing the counts, measured in wells with different stimulators and culture conditions, by the counts produced by unstimulated responder cells, cultured in medium alone.

Generation of MCL-reactive CTL lines and clones

MNC from two unrelated healthy HLA class I-matched donors at a concentration of 10^6 cells / well in 6-well plates (Costar) were stimulated with irradiated (30 Gy) primary MCL cells, or CD40-activated MCL cells at responder/stimulator (R/S) ratios of 10:1 in medium consisting of IMDM (BioWittaker) and 10 % human serum. IL-2 (Chirion, Amsterdam, the Netherlands) at a final concentration of 100 U/mL was added at day 6. Twice weekly, half of the medium was refreshed with IL-2 containing medium. At day 9 the T cells lines were harvested, and depleted of CD4⁺ T cells using anti-CD4-conjugated magnetic beads (Milteny Biotec, Bergisch Gladbach, Germany) since the donors were HLA class II-mismatched. The T cell lines were restimulated with irradiated stimulator cells at the same R/S ratios at days 9, 16 and 23. T cells were harvested 5-7 days after the third or fourth stimulation for phenotypic analysis, and used as effectors in cytotoxicity assays. To generate MCL-reactive CTL clones, the donor-derived leukemia-reactive CTL lines were plated at frequencies of 3, 1 and 0.3 cells/well in 96-well microtiter

plates, and expanded in the presence of irradiated allogeneic feeder cells (5×10^4 cells / well) in medium consisting of IMDM plus 10% human serum, IL-2 (100 U /mL) and phytohemagglutinin (PHA, 800 ng/mL, Murex Biotech Limited, Dartfort, UK). When sufficient cell numbers were reached T cell clones were harvested, and tested for specific cytolytic activity against the primary MCL cells, CD40 activated MCL cells, and PHA blasts from patient and donor. As read-out system for T-cell-mediated cytotoxicity, our recently developed carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytotoxicity assay was used ³⁵.

CFSE-based cytotoxicity assay

The CFSE-based cytotoxicity assay facilitates the quantitative analysis of susceptibility to T-cell-mediated lysis of malignant cell types within a heterogeneous target cell population. Briefly, after washing with phosphate-buffered saline (PBS) the target cells were resuspended at 20×10^6 cells/mL and labeled with 10 μ M CFSE (Molecular Probes Europe, Leiden, the Netherlands) for 10 minutes at 37°C. The reaction was stopped by the addition of an equal volume of fetal calf serum (FCS), followed by a 2-minute incubation at room temperature. After 2 washes the CFSE-labeled target cells were resuspended in culture medium at a cell concentration of 5×10^4 cells/mL, and 100 μ L/well was plated in 96-well microtiter plates. CTLs were added at different effector/target (E/T) ratios ranging from 0.3:1 to 10:1. After 5 hours and 12 hours of incubation in a humidified atmosphere of 5% CO₂ and 37°C the wells were harvested, and to allow quantitative analysis of the cell populations 10,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL) were added. To stain for dead cells, propidium iodide (0.5 μ g/mL) was added, samples were mixed properly, and directly analyzed by flow cytometry. For each sample 5,000 microbeads were acquired and the absolute number of surviving cells was determined at each time point by calculation of the ratio between the number of viable cells and the number of beads. The percentage of survival was calculated as follows: % survival = [absolute # viable CFSE⁺ target cells (t = x)]/[absolute # viable CFSE⁺ target cells (t = 0)] x 100. Percentage of specific lysis was defined as: 100% - % survival. To analyze the HLA class-I or -II restriction of the target cell lysis blocking studies were performed. Target cells were incubated with saturating concentrations of anti-HLA class I or anti-HLA class II MoAb (W6/32 and PdV5.2, respectively) for 30 minutes before effector and target cells were cocultured. Blocking experiments at effector level were performed by adding anti-CD8 MoAb (FK18, RIVM, Bilthoven, the Netherlands) at a final dilution of 8 μ g/mL 30 minutes prior to the addition of target cells.

Statistical analysis

Statistical significance of differences was determined by the paired two-tailed Student's *t*-test or by the Wilcoxon test using the absolute values.

Results

Expression and upregulation of adhesion and costimulatory molecules on MCL cells.

To determine cell surface expression of the adhesion molecules CD54 and CD58, and the costimulatory molecules CD40, CD80, CD86 and CD83, immunophenotyping of freshly isolated MCL cells from 6 patients was performed.

Table 3. Phenotype of isolated MCL cells.

Patient	Recognition		Adhesion		Costimulation			
	MHC II		CD54	CD58	CD80	CD86	CD83	CD40
MFIR (% positive cells)								
MCL 1	+++	(97)	+ (42)	++ (26)	± (13)	0 (4)	0 (9)	++ (95)
MCL 2	+++	(97)	++ (37)	0 (6)	± (11)	± (10)	0 (2)	+ (91)
MCL 3	+++	(94)	+ (83)	+ (93)	± (52)	0 (3)	0 (2)	++ (88)
MCL 4	+++	(99)	+ (13)	+ (29)	+ (84)	0 (3)	+ (20)	++ (88)
MCL 5	+	(72)	+ (14)	+ (15)	0 (2)	0 (5)	0 (2)	++ (83)
MCL 6	+++	(96)	+ (17)	++ (28)	0 (7)	0 (1)	0 (7)	++ (96)

Mean fluorescence intensity ratio (MFIR) is mean fluorescence intensity (MFI) of cells stained with a fluorochrome-conjugated antigen-specific MoAb divided by MFI of cells stained with a fluorochrome-conjugated isotype-control MoAb. If the percentage of positive events was <10 %, MFIR of the leukemic sample was not calculated and is expressed as 0. If the percentage of positive events was ≥ 10 % then the MFIR is calculated and depicted as: ± < 10; + 10 – 20; ++ 20 – 50; +++ 50 – 100

As shown in table 3, the MCL cells of all patients strongly expressed HLA class II (MFIR >50). In most patients (n=5), a minority of the MCL cells (<45%) expressed CD54 at low levels (MFIR <20). One patient (MCL 2) had intermediate levels (MFIR 20-50) of CD54 expression, but lacked CD58 expression. In MCL 3, almost the entire population (>80%) expressed the adhesion molecules but expression levels were low. Two out of six patients showed intermediate levels of CD58. In only one patient (MCL 4) some expression of CD80 and CD83 was observed, all other MCL cases expressed no or neglectable levels of CD80, CD86 and CD83. Only the costimulatory molecule CD40 was expressed by all MCL cells at intermediate levels. In summary, in accordance with other B cell malignancies, inadequate expression of costimulatory molecules on MCL cells was observed.

To upregulate the expression of adhesion and costimulatory molecules on MCL cells, we first examined several cytokines, including IFN- α , TNF- α , IL-4 and IL-10. Next, activation through TLR 9 by its synthetic ligand CpG ODN 2006 in combination with the various cytokines was tested. Finally, CD40 engagement as stimulus to modify MCL cells into APCs was analyzed in the presence of cytokines and/or CpG. Figure 1 summarizes the FACS analysis data of the most optimal combinations

to upregulate adhesion and costimulatory molecules on MCL cells. The data are presented as percentages of positive MCL cells (figure 1A) and the MFIR (figure 1B), and were obtained after a culture period of 96 hours.

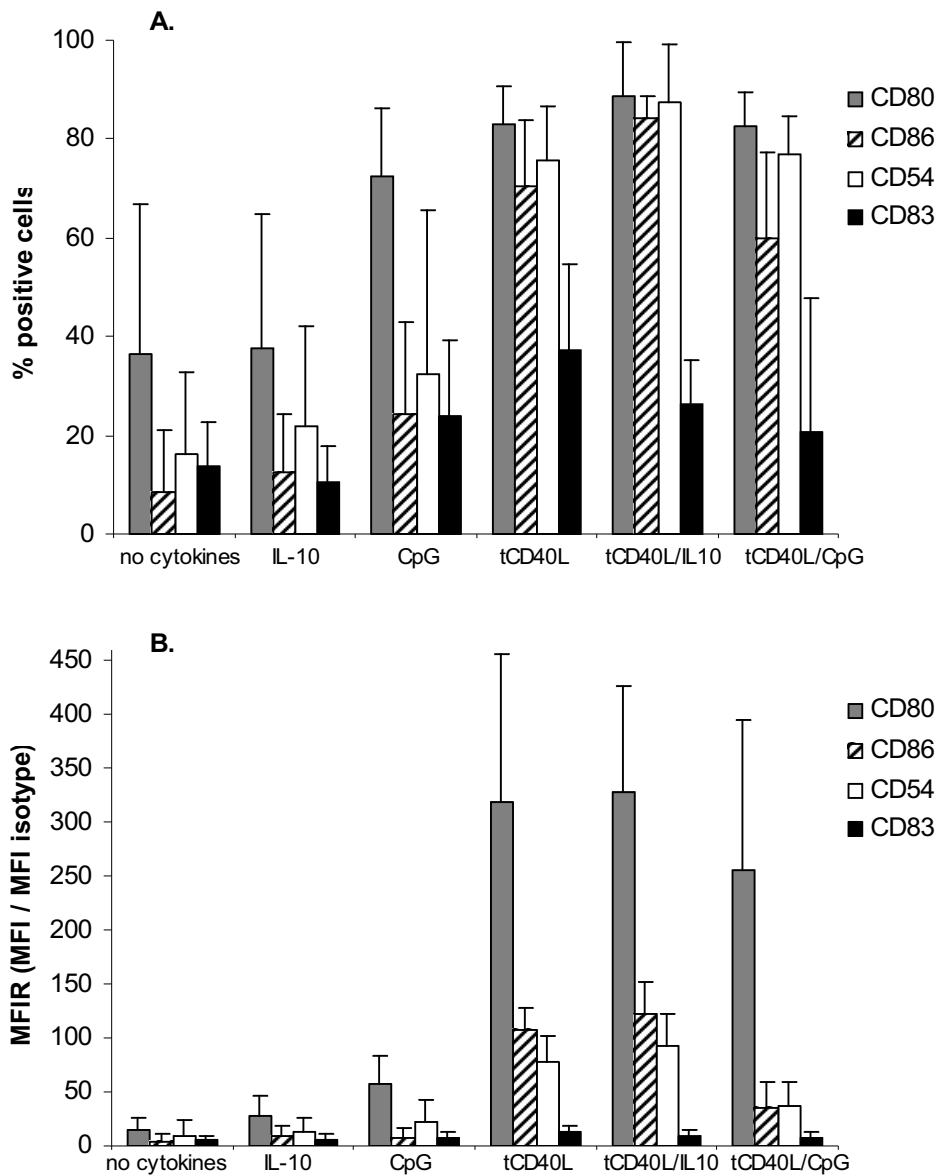


Figure 1: Expression and upregulation of costimulatory and adhesion molecules on MCL cells activated by IL-10, CpG and/or tCD40L. (A) Combinations using CD40 activation give significant higher percentages of positive MCL cells for CD80 and CD86 ($p < 0.01$) (B) Stimulation of MCL cells by tCD40L gives maximal upregulation of expression levels of CD80 and CD86. MCL cells were cultured for 96 hours in the presence or absence of IL-10 (10 ng/mL), CpG (10 μ g/mL) and/or tCD40L. Expression of CD80, CD86, CD54 and CD83 was analyzed by flow cytometry. Mean fluorescence intensity ratio (MFIR) was calculated as described in Material and Methods section, only if percentage of positive cells was $> 10\%$. Results are expressed as mean \pm SD of 6 patients with MCL.

None of the tested cytokines caused any upregulation. CpG increased the percentage of MCL cells positive for costimulatory molecules tested, but only the expression level of CD80 was enhanced. Using flowcytometry, only weak expression of TLR9 was observed (data not shown). Accordingly, only

minor changes were observed after stimulation of MCL cells with CpG. CD40 activation by tCD40L significantly increased the percentage of positive MCL cells for all adhesion and costimulatory molecules ($p < 0.01$). As demonstrated in figure 1B, the expression levels of all these molecules, especially CD80 (MFIR 21.3 fold, $p = 0.001$) on MCL cells were strongly upregulated, and transformed MCL cells into characteristic APC phenotypes with high expression of CD80, CD86 and CD54. Additional stimulation with CpG or IL-10 did not further enhance the upregulation.

To investigate the optimal time period of stimulation for full activation of MCL, phenotypic analysis of MCL cells of all six patients was performed 2, 4 and 6 days after stimulation with tCD40L. As shown in figure 2, CD40 activation of MCL cells by tCD40L caused strong upregulation of CD80, CD86, and CD54 within 2 days. After 4 days of stimulation a further enhancement of expression levels of CD80 (MFIR 2.9 fold, $p = 0.01$) and CD86 (MFIR 3.5, $p = 0.02$) was observed. Although a significantly higher percentage of leukemic cells expressed CD83 after CD40 stimulation, the expression levels of CD83 were only minimally upregulated.

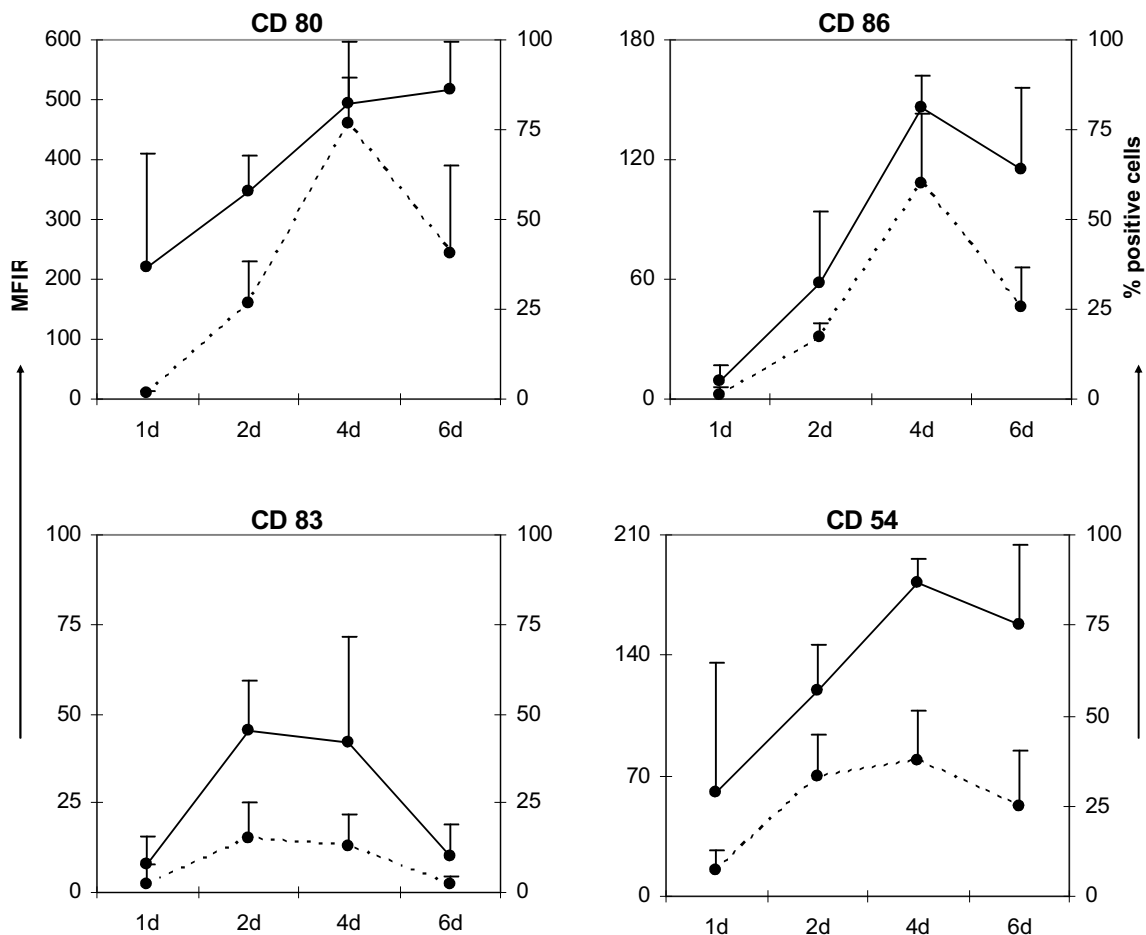


Figure 2. Kinetics of upregulation of costimulatory molecules on MCL cells activated by tCD40L. MCL cells were activated by tCD40L. MFIR and percentage of MCL cells positive for CD80, CD86, CD83 and CD54 were analyzed at different time points by flow cytometry. Data are shown as means \pm SD for 6 patients. Straight lines indicate % positive cells, dotted lines represent MFIR.

Cytospin preparations of both unstimulated and tCD40L-activated MCL cells showed that after CD40 triggering, MCL cells increased 2-fold in size and obtained a dendritic cell-like morphology with a high degree of vacuolization. In conclusion, in the absence of CD40 activation minimal upregulation was observed in response to IL-10 and CpG. Stimulation of MCL cells by tCD40L for a period of 4 days caused the strongest upregulation of costimulatory and adhesion molecules, and modified MCL cells into morphological and phenotypical characteristic APC.

To examine the functional capacity of the different MCL APC to produce IL-10 and IL-12, supernatants from cultures of activated MCL cells after 96 hours were measured. Whereas unstimulated MCL and CpG activated MCL cells were not capable of producing significant amounts of IL-12 (median <15 pg/mL), high levels of IL-12 were produced by tCD40L-activated MCL cells with major variability between different patients (median 1,054 pg/mL; range 67-8,800 pg/mL, n=6). Neglectable amounts of IL-12p70 (mean 6 pg/mL) and IL-10 (mean 14 pg/mL) were produced by the CD40-activated MCL cells. Additional stimulation with CpG induced some increase in IL-12 production (median 1,370 pg/mL) and IL-12p70 (mean 31 pg/mL) but also induced the production of IL-10 (mean 299 pg/mL).

MLR using primary or MCL-APC as stimulator cells in the presence or absence of IL-10 and IL-12

To investigate the functional significance of IL-12 or IL-10 on the stimulatory capacity of MCL cells, a MLR was performed using primary MCL cells or MCL-APC as stimulator cells, and MNC from a donor as responder cells in medium with or without IL-10 or IL-12. As expected, primary MCL cells, derived from three different patients, had minimal allostimulatory capacity (see figure 3). IL-12 had a weak stimulatory effect on the proliferation of the allogeneic T cells, whereas IL-10 demonstrated some inhibition of T cell activation. In contrast, the stimulation index in response to MCL-APC as stimulators, was 25-50, illustrating the impressive immunostimulatory capacity of these malignant APC. As shown in figure 3, further increase of IL-12 concentrations did not enhance T cell proliferation nor did IL-10 suppress the induction of proliferation. In conclusion, tCD40L-activated MCL cells were considered the best MCL-APC, and were further used as stimulator cells in the subsequent experiments.

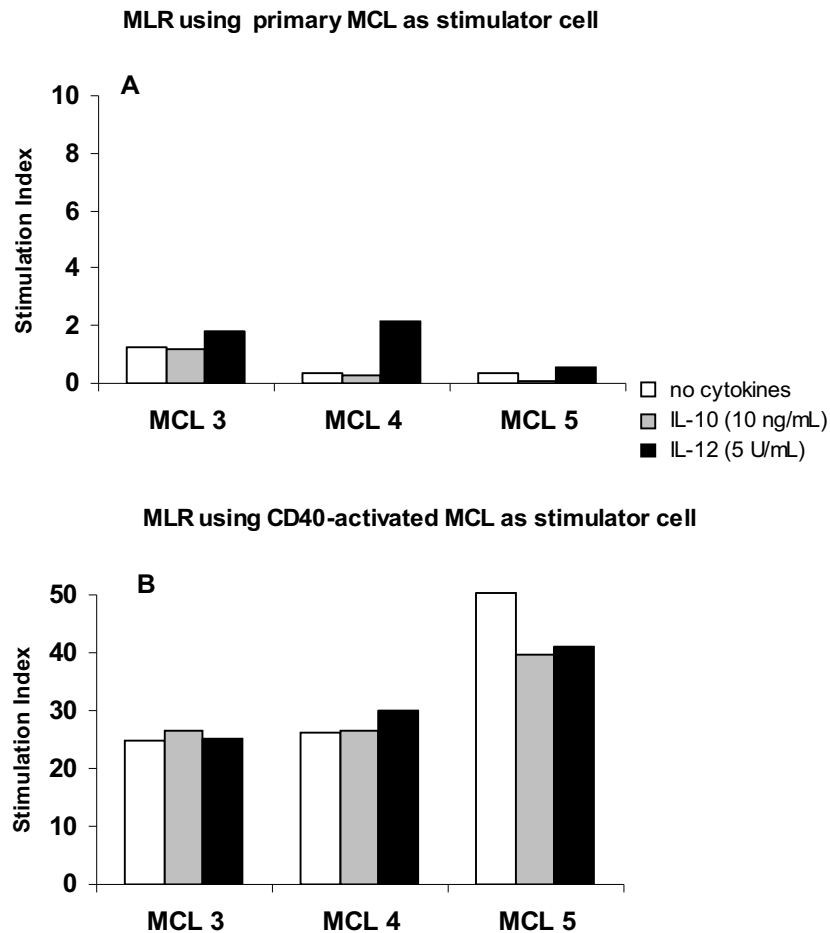


Figure 3. The proliferation and cytotoxicity of allogeneic CD8⁺ T cells in response to primary MCL and MCL-APC cells. (A) In contrast to primary MCL, MCL-APC cells as stimulators induced strong proliferation of CD8⁺ donor T cells in the URD anti-MCL-APC 3 and URD anti-MCL-APC 4 combinations. (B) cytotoxic activity of the CTL lines generated against MCL-APC 3 or MCL-APC 4 measured in a 12-hours CFSE-based cytotoxicity assay.

The generation of MCL-reactive CTL lines and clones from HLA-matched donors

To analyze the antigen-presenting capacity of primary MCL cells and tCD40L-activated MCL (MCL-APC) T cells from a fully HLA class I-matched donor and an unrelated HLA-A- and HLA-B-matched donor were stimulated with primary MCL or MCL-APC cells. CD4 depletion was performed at day 9 to eliminate allo-HLA class II responses. As demonstrated in figure 4, primary MCL cells were not capable of inducing T-cell proliferation and generating T cell lines. The cytotoxic activity of the CTL lines generated against MCL-APC was measured using the CFSE-based cytotoxicity assay³⁵ (see Material and methods) with CFSE-labeled primary MCL cells, MCL-APC cells, and PHA blasts from the patients and donors as target cells. As illustrated in figure 4, the CD8⁺ CTL lines derived from the donors effectively killed the primary MCL cells, the MCL-APC, the PHA blasts from the patient, and not the PHA blasts from the donor in a 12 hours CFSE cytotoxicity assay.

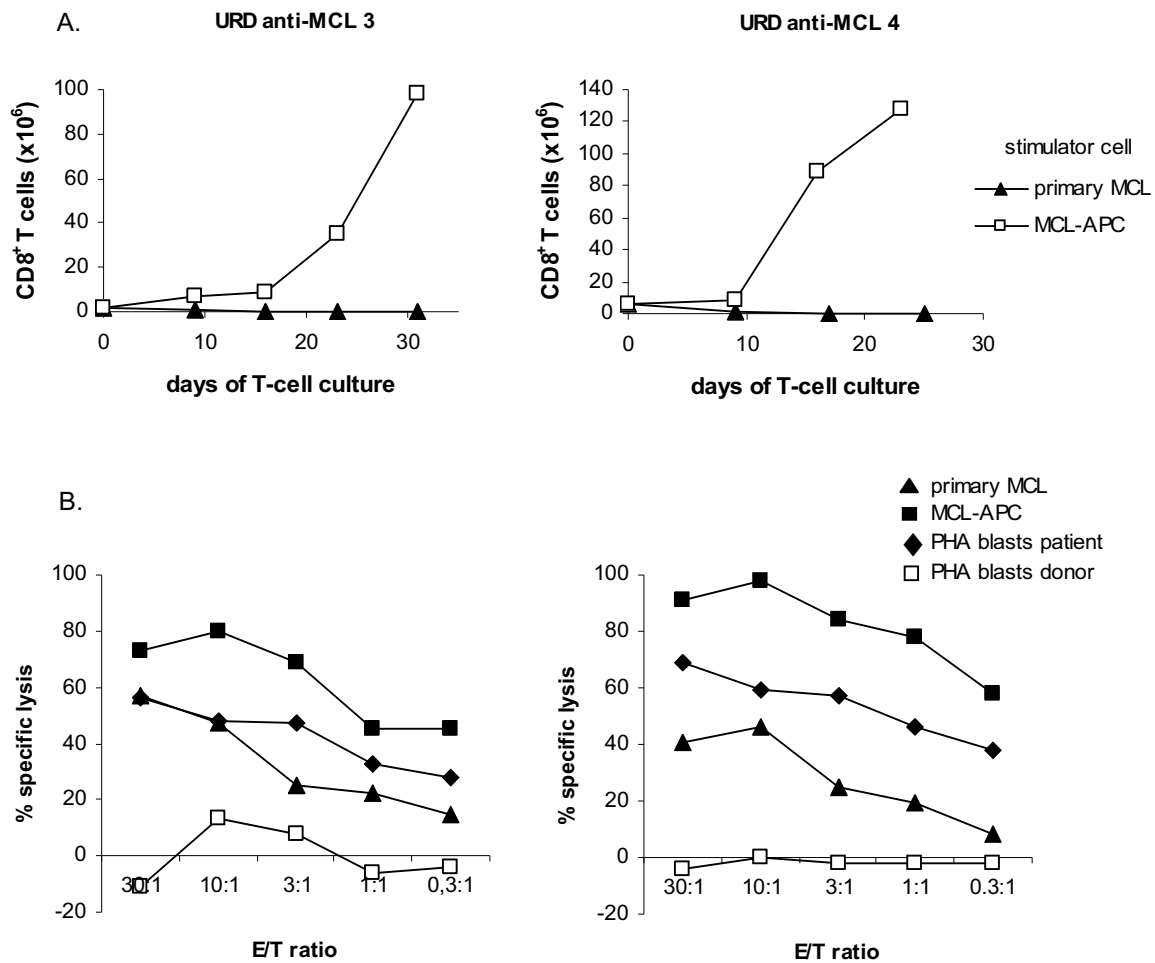


Figure 4. The proliferation and cytotoxicity of allogeneic CD8⁺ T cells in response to primary MCL and MCL-APC cells. (A) In contrast to primary MCL, MCL-APC cells as stimulators induced strong proliferation of CD8⁺ donor T cells in the URD anti-MCL-APC 3 and URD anti-MCL-APC 4 combinations. (B) cytotoxic activity of the CTL lines generated against MCL-APC 3 or MCL-APC 4 measured in a 12-hours CFSE-based cytotoxicity assay. Although there was an HLA-C locus mismatch in the MCL 4/donor combination and although the primary MCL 4 had some expression of CD80 and CD83 (see table 2) this was insufficient to overcome T cell anergy and to induce antigen-driven proliferation. In contrast, using MCL-APC cells as stimulators, vigorous expansion of CD8⁺ T cells was observed in both patient/ donor combinations tested.

To estimate the precursor frequency of MCL-reactive T cells in the CTL lines, and to determine whether the cytotoxicity of the CTL lines was exerted by cytotoxic T cells with different specificities CTL clones were generated. In the URD α -MCL-APC 3 combination 13% of the 1 cell/well and 8% of the 0.3 cell/well showed proliferation. 29 out of 91 proliferating CD8⁺T cell clones showed specific lysis of primary MCL at an E/T ratio of 10:1 ($36 \pm 14\%$, mean \pm SD, n=29) in a 12 hours CFSE cytotoxicity assay. The plating efficiency in the second combination (URD α -MCL-APC 4) was 12%, resulting in 60 out of 89 CD8⁺CTL clones, recognizing primary MCL cells ($65 \pm 20\%$, mean \pm SD, n=60). These results indicated that in both CTL lines high frequencies of MCL-reactive T cells were present.

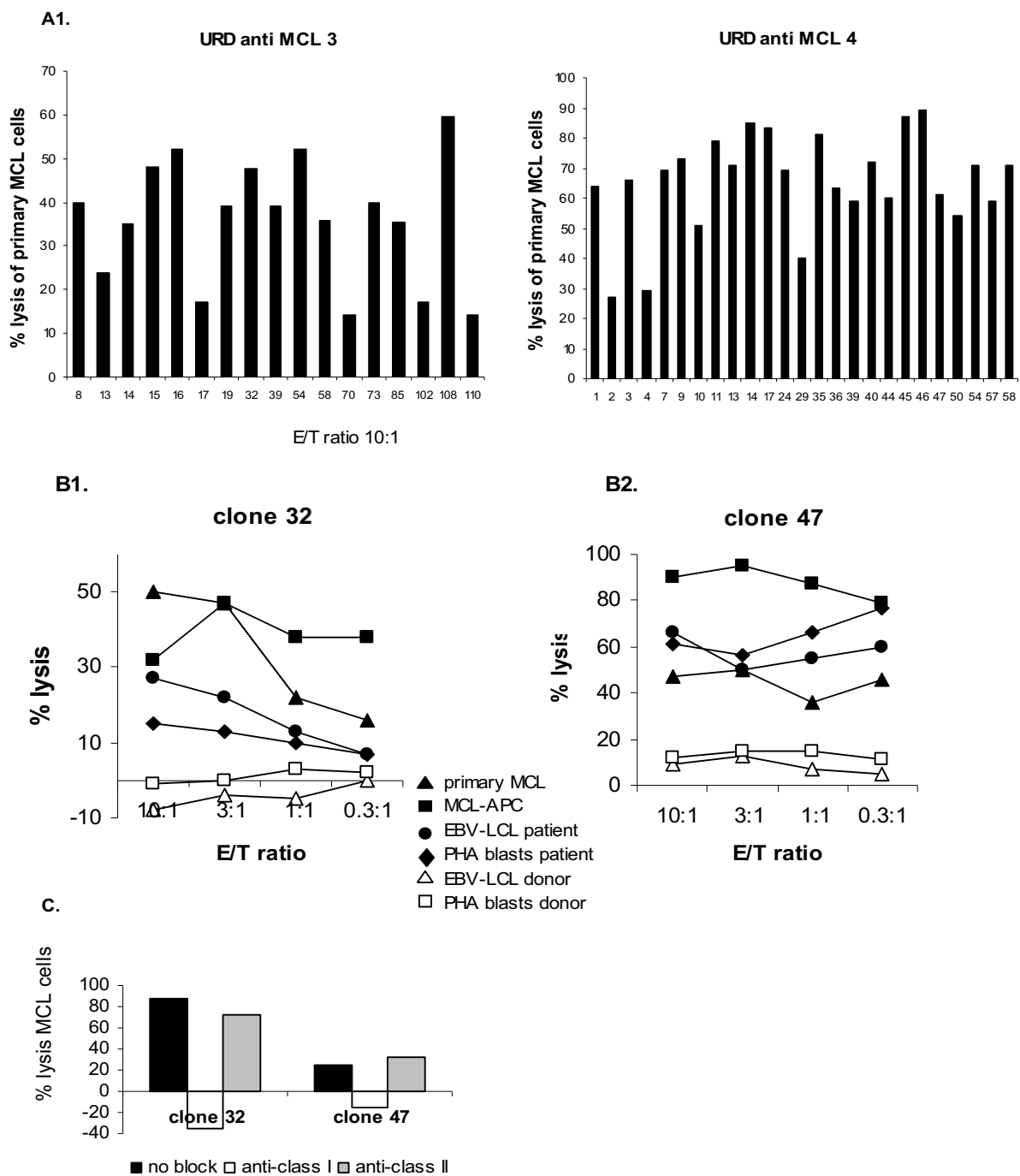


Figure 5. Cytotoxicity and specificity of representative MCL-reactive CD8+ CTL clones, derived from HLA-matched donors. (A1 and A2.) High numbers of CD8+ CTL clones recognizing primary MCL in a 12-hours CFSE cytotoxicity assay were generated from both URD (B1. and B2.) Four representative CTL clones from each donor/patient pair were analyzed for specific cytolytic activity against MCL, MCL-APC, and PHA blasts from donor and patient in a 12-hours CFSE cytotoxicity assay. All clones recognized MCL-specific as well as patient-derived targets and not PHA blasts or EBV-LCL from the donor. One representative example of these CTL clones from each combination is demonstrated. (C.) HLA class I-restriction of the cytotoxicity was demonstrated by blocking experiments using primary MCL as target at an E/T ratio of 10:1. Target cells were 30 minutes preincubated with anti-HLA class I (W6/32) or anti-HLA class II (PdV5.2) antibodies prior to the CTL assay using clone 32, derived from URD 3 and clone 47, derived from URD 4 as effector cells.

To analyze whether the MCL-reactive CTL clones were MCL-, B cell- or mHag-specific, four representative clones in both patient/donor pairs were tested for cytotoxicity against MCL-specific targets, patient- and donor-derived T lymphoblasts (PHA blasts) and B-cell-specific targets (EBV-LCL) from patient and donor. As shown in figure 5, CTL clones generated from the CTL lines from both donors were reactive against MCL, MCL-APC, and EBV-LCL of the patient, and not against donor-specific targets. The clones also killed T cell-derived targets indicating that these T cell clones were probably mHag-specific and that the recognized mHag is not B-lineage-restricted. To confirm HLA class I-restricted recognition of the targets by the CD8⁺ CTL lines and clones, and to exclude HLA class II-restricted killing by contaminating CD4⁺ T cells, blocking studies were performed using representative examples of the generated MCL-reactive CTL lines and clones. Cytotoxicity of both CTL lines was completely blocked by anti-HLA class I or anti-CD8 antibodies and not by the addition of anti-HLA class II (data not shown). Cytolytic activity of the CTL clones could partially or completely be abrogated using the anti-HLA class I antibodies (figure 5).

Discussion

RIC allogeneic SCT is considered as a new promising treatment modality for patients with advanced MCL. After allogeneic SCT adoptive transfer of donor T cells, capable of killing MCL cells may eliminate residual malignant cells resulting in long-term remissions. In this context, this experimental study was performed to evaluate whether donor T cells can be triggered to preferential kill MCL-specific targets using MCL cells as stimulator cells. As illustrated in the results, we demonstrated that primary MCL cells as stimulator cells failed to induce proliferation and to generate a T-cell response, even in the presence of IL-12, probably due to the lack of expression of costimulatory molecules. Thus, to generate donor T cells with high avidity for MCL cells, modification of the MCL cells into professional APC with high expression of costimulatory and adhesion molecules was hypothesized to be essential. Treatment of MCL cells with proinflammatory cytokines, B-cell activating cytokines, and also the MCL-stimulating cytokine IL-10 did not significantly induce phenotypic changes of the MCL cells. In accordance with a recent report³⁶, we observed limited activation of MCL cells upon stimulation with CpG ODN 2006, probably due to the low expression of TLR9 on MCL cells.

Since CD40 engagement is the major signal that induces B cells to efficiently present antigen to T cells^{16,18,37}, and since malignant B cells strongly express CD40, activation through CD40 can be an effective tool to transform tumor B cells into an antigen-presenting phenotype^{15,17,20,27,28}. In the present study, we show that CD40-ligation could modify MCL cells into phenotypically professional APCs with high expression of CD80, CD86, and the adhesion molecules, and induced some expression of CD83. Additional stimulation through TLR9 and cytokine receptors did not further enhance expression levels. In contrast with recent cytokine gene expression studies suggesting that both primary as well as CD40-activated MCL cells cannot produce IL-12p40 and assemble the IL-12 heterodimer³⁸, we illustrated in the present study the capacity of MCL cells to synthesize IL-12. MCL cells were only capable of producing IL-12 in response to CD40 stimulation. The MLR experiments

demonstrated that using MCL-APC as stimulator cells an increase in IL-12 concentrations did not further enhance T-cell proliferation.

Next, the stimulatory capacity of the generated MCL-APC to induce vigorous allogeneic MCL-specific T-cell responses was compared to primary MCL as stimulator cell in two allogeneic donors. In the HLA-C locus mismatched setting (URD α MCL 4) the unmodified MCL cells as stimulators did not induce any antigen-driven proliferation. Although MCL 4 showed some expression of the costimulatory molecules, this was apparently insufficient to induce an appropriate T-cell response over a major MHC mismatch barrier. In contrast, the MCL-APCs were capable of provoking vigorous expansion of the donor-derived T cells and of eliciting a sustained primary allogeneic MCL-reactive immune response. These results illustrate that primary MCL cells can be adequately killed by T cells generated against MCL-APCs. To evaluate whether MCL-specific CD8⁺ T-cell responses could be generated in a fully HLA-class I-matched setting MNC from the URD were stimulated with primary MCL 3 or MCL 3-APC. Again no T-cell proliferation was observed against primary MCL and cytotoxic MCL-reactive CD8⁺ T-cell lines and clones could be generated against MCL-APC, indicating that MCL-APCs are essential to induce an efficient anti-leukemic T-cell response. Allo-HLA-class II responses were eradicated by CD4 depletion and blocking experiments confirmed HLA class I-restricted recognition of the target cells. To further elucidate the different specificities of the leukemia-reactive T cells and to determine the precursor frequencies of these T cells in the CTL line, cloning experiments were performed. This resulted in high clonal expansion efficiency of 6-18% of the cells isolated, suggesting that MCL-APCs as stimulator cells enrich for MCL-reactive T cells. MCL-specific targets, but also patient-derived targets from T-cell (PHA blasts) and B-cell origin (EBV-LCL) were recognized by the CTL clones, indicating that the immunogenic antigen is not MCL- or B-cell-specific, but most likely mHag-specific. As we previously illustrated for patients with acute leukemia's^{14,39,40}, repeated stimulation of donor T cells with leukemic cells results in the generation of T-cell responses against mHag with relative specificity for the malignant cells. Therefore, although other patient-derived APC may be capable of eliciting mHag specific T-cell responses, MCL-derived APC likely will skew the immune response toward recognition of MCL-specific antigens or mHags highly expressed on the MCL cells thus leading to a more specific and efficient T-cell response.

In summary, our study shows that T cell anergy to primary MCL cells can be reversed by transforming MCL cells into professional malignant APCs using CD40 ligation and provides the first evidence that using these MCL-APCs as stimulators MCL-reactive mHag-specific CD8⁺ CTL lines and clones can be readily generated from MNC of HLA class I-matched donors. Two recent reports demonstrated the feasibility of RIC allogeneic SCT as salvage therapy in patients with advanced MCL^{41,42}. After allogeneic SCT sensible RT-PCR techniques for minimal residual disease and donor chimerism analysis enable to predict disease recurrence. The administration of DLI can lead to long-term remissions, but effectiveness may be limited due to low immunogenicity of MCL cells and the development of acute or chronic GvHD, caused by non-selected donor T cells. In this setting the application of mHag-specific CTLs, which preferentially kill MCL cells and not non-hematopoietic targets

¹⁴ could be of great value and could prevent or treat disease recurrence after allogeneic SCT. The magnitude and specificity of the immune response against MCL cells without causing concomitant GvHD may be maximized.

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