Leukemia-Specific T-Cell Reactivity Induced by Leukemic Dendritic Cells Is Augmented by 4-1BB Targeting

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Abstract

Purpose: Acute myelogenous leukemia (AML) blasts are able to differentiate into leukemia-derived dendritic cells (AML-DC), thereby enabling efficient presentation of known and unknown leukemic antigens. Advances in culture techniques and AML-DC characterization justify clinical application. However, additional measures are likely needed to potentiate vaccines and overcome the intrinsic tolerant state of the patients' immune system. Engagement of the costimulatory molecule 4-1BB can break immunologic tolerance and increase CTL responses. In this study, we examined the role of the 4-1BB ligand (4-1BBL) on T-cell responses induced by AML-DC.

Experimental Design: In allogeneic and autologous cocultures of T cells and AML-DC, the effect of the addition of 4-1BBL on T-cell proliferation, T-cell subpopulations, and T-cell function was determined.

Results: Addition of 4-1BBL to cocultures of AML-DC and T cells induced a preferential increase in the proliferation of $CD8^+$ T cells. Increased differentiation into effector and central memory populations was observed in both $CD4^+$ and $CD8^+$ T cells in the presence of 4-1BBL. AML-DC induce a T helper 1 response, characterized by high IFN-γ production, which is significantly increased by targeting 4-1BB. T cells primed in the presence of 4-1BBL show specificity for the leukemia-associated antigen Wilms' tumor 1, whereas cytotoxicity assays with leukemic blast targets showed the cytolytic potential of T cells primed in the presence of 4-1BBL.

Conclusion: We conclude that 4-1BBL is an effective adjuvant to enhance T-cell responses elicited by AML-DC.

Efficient T-cell activation by dendritic cells requires at least two signals (1). The first signal controls the specificity of the T-cell response through the presentation of epitopes in the context of the MHC to the T-cell receptor (TCR). The second signal, termed costimulation, is mandatory to induce full T-cell activation and is provided by a number of receptor-ligand interactions (2). It is well established that initial T-cell activation is dependent on the interaction of CD28 with the costimulatory molecules CD80 and CD86. Several molecules of the tumor necrosis factor receptor family (e.g., 4-1BB) act as costimulatory molecules that enhance and maintain the immune response subsequent to initial T-cell activation (3).

4-1BB is an inducible costimulator that is present on activated T cells. Besides activated T cells, natural killer (NK) cells and dendritic cells also show 4-1BB expression. On dendritic cells, engagement of 4-1BB can induce interleukin (IL)-6 and IL-12 production (4, 5). 4-1BB ligand (4-1BBL) is expressed on activated macrophages, dendritic cells, and B cells. Engagement of 4-1BB on activated T cells is associated with enhanced proliferation and protection from activation-induced cell death, with profound effects on the $CD8^+$ T-cell population and modest effects on the $CD4^+$ T-cell population. Targeting 4-1BB increases effector functions of activated T cells and has been shown to contribute to antitumor immunity (6–10). These findings reveal a critical role for 4-1BB in the expansion of functionally active CTL. Interestingly, humanized single-chain variable fragment anti-4-1BB is now available for clinical trials (11).

In the search for new treatment modalities aiming at eradicating minimal residual disease in acute myelogenous leukemia (AML), immunotherapy seems an attractive option. Leukemic blasts have proven to be able to differentiate into AML-derived dendritic cells (AML-DC), thereby maintaining leukemia-specific antigens and obtaining full capacity to present these antigens (12–14). *In vitro* studies confirmed the ability of AML-DC to migrate toward lymph node–associated chemokines to induce T-cell proliferation and, most importantly, to induce leukemia-specific cytotoxicity (15–17). Thus, the vaccination of AML patients with autologous AML-DC could potentially enhance leukemia-specific T-cell responses and prevent outgrowth of minimal residual disease cells.

Although preclinical data regarding the use of dendritic cells for antitumor immune responses are encouraging, clinical studies have shown only limited success (18, 19). It is now

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thought that additional measures to potentiate the immune response induced by dendritic cells are needed to augment clinical efficacy of the dendritic cell vaccination approach. We observed that mature AML-DC lack the expression of 4-1BBL. This prompted us to study the effect of 4-1BBL on T-cell responses induced by repeated stimulation with leukemia-derived dendritic cells. We show that targeting of 4-1BB enhances T-cell proliferation and differentiation with increased IFN-γ production. Our results indicate that the engagement of 4-1BB could provide a valuable method to strengthen the immune response induced by leukemic dendritic cells.

Materials and Methods

Patients and healthy donors. Blood or bone marrow samples of patients either with newly diagnosed AML or in complete remission and healthy donors were obtained after informed consent.

Preparation of leukemic dendritic cells. AML-DC were generated from fresh or thawed mononuclear cells of AML samples in CellGro serum-free culture medium (CellGenix, Freiburg, Germany) using granulocyte macrophage colony-stimulating factor, tumor necrosis factor-α, stem cell factor, Flt-3L, IL-3, and IL-4 or calcium ionophore A23187 in combination with IL-4 as described previously (14, 16). Maturation of cytokine-cultured AML-DC was induced by an additional 48 hours of culture with tumor necrosis factor-α, IL-1β, IL-6, and prostaglandin E_2 as previously described (15). Because no functional differences between calcium ionophore–cultured and mature cytokine–cultured AML-DC could be established previously, the culture method is not further specified in the functional experiments (15). The leukemic origin of AML-DC was previously established by fluorescence *in situ* hybridization analysis (14). Cell number and viability were determined by trypan blue dye exclusion. The percentage of viable, apoptotic, and necrotic cells was evaluated by flow cytometry using Syto 16 (3 nmol/L, Molecular Probes, Eugene, OR) and 7-amino-actinomycin D (ViaProbe, BD PharMingen, San Diego, CA; ref. 20).

Isolation of T-cell subpopulations. Mononuclear cells from healthy donors or AML patients in complete remission were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). Monocytes were depleted by plastic adherence. Naïve T cells were obtained by labeling the cells with the monoclonal antibodies CD16, CD19, and CD45RO, and by isolating them with goat anti-mouse microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) through immunomagnetic column separation using negative depletion. In some experiments, $CD8^+$ cells were separated from $CD8^-$ cells by immunomagnetic column separation using $CD8^-$ microbeads (Miltenyi Biotec).

Cocultures of AML-DC and T cells. Irradiated (30 Gy) AML-DC were cocultured with either the complete allogeneic lymphocyte population ($n = 4$), allogeneic naïve T-cell population ($n = 3$), or autologous $CD8^+$ T-cell population ($n = 4$) at a 1:5 ratio in RPMI 1640 (Life Technologies, Paisley, Scotland), 5% human serum (BioWhittaker, Walkersville, MD), 50 μmol/L β-mercaptoethanol (Sigma, St. Louis, MO), 100 units/mL penicillin (Life Technologies), and 100 μg/mL streptomycin (Life Technologies). Autologous cocultures were initiated with the $CD8^+$ T-cell population, and irradiated (30 Gy) $CD8^-$ T cells were added as feeders at 1:1 ratio to provide additional T-cell help (21). Cocultures were restimulated weekly with fresh, irradiated AML-DC. IL-7 (5 ng/mL; specific activity, 5×10^7 units/mg of protein; Strathmann Biotec, Hamburg, Germany) was added at the start of cocultures and upon each subsequent restimulation. At day 2 and 2 days after each restimulation, IL-2 (10 units/mL; specific activity, 1×10^7 units/mg protein; Strathmann Biotec) was added. 4-1BBL (10 ng/mL; PeproTech, Rocky Hill, NJ) was added at the start of the coculture and during subsequent restimulations. At the end of the coculture period, the culture supernatant was harvested and stored for cytokine analysis by ELISA. Each week, cell counts were obtained by trypan blue dye exclusion, and samples were taken and analyzed by flow cytometry.

Immunophenotypic analysis. Before and after culture, AML blasts and AML-DC were characterized by four-color, flow cytometric analysis on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using the following monoclonal antibodies: FITC-labeled CD86 (PharMingen), CD1a (Sanquin, Amsterdam, the Netherlands), CD54 (DAKO, Glostrup, Denmark), phycoerythrin-labeled CD40 (Immunotech, Marseilles, France), CD80, CD83 (Immunotech), CD137 (PharMingen), CD137L (PharMingen), peridinin chlorophyll-α protein–labeled CD45, anti-HLA-DR, allophycocyanin-labeled CD34, CD38, and CD14.

T-cell subpopulations were analyzed before, during, and after cocultures using FITC-labeled CD28 (DAKO), CD45RA (Sanquin), CD45RO (DAKO), phycoerythrin-labeled CD4, CD27, CD137 (PharMingen), antihuman IFN-γ, antihuman IL-4, peridinin chlorophyll-α protein–labeled CD8, and allophycocyanin-labeled CD3. Unless stated otherwise, monoclonal antibodies were purchased from Becton Dickinson.

HLA-A201 restricted Wilms' tumor 1 (WT-1) dextramers [RMFPNAPYL (126–134), DAKO] were used to analyze T-cell specificity before and during cocultures with $HLA\text{-}A2^+$ T cells.

Flow cytometric analysis of TCR Vβ expression was done using antibodies present in the β mark TCR Vβ repertoire kit before and during cocultures (Beckman Coulter, Marseilles, France).

Cytokine analysis. Coculture supernatants were analyzed for IL-4, IL-10, and IFN-γ using ELISA kits (Sanquin) according to instructions of the manufacturer.

Cytotoxicity assay. The ability of cultured T cells to kill leukemic blasts was evaluated in a flow cytometric cytotoxicity assay, as previously described (17). Briefly, AML-DC–stimulated T cells (effector cells) with or without 4-1BBL were cultured with corresponding AML blasts (target cells) at different effector to target cell ratios. After 6 hours of culture, T cells and AML blasts were stained with a specific T-cell marker (allophycocyanin-labeled CD3) and a specific AML blast marker (phycoerythrin-labeled CD33). Syto 16/7-amino-actinomycin D staining identified early apoptosis and secondary necrosis. MHC restriction of the cytotoxic response was analyzed by using an MHC class I–blocking antibody (W6.32, 2.5 μg/mL, a kind gift of Dr. S.M. van Ham, Department of Immunopathology, Sanquin Research at CLB, Amsterdam, the Netherlands) and its appropriate isotype control (mouse IgG2a, 2.5 μg/mL, Sanquin).

Statistics. Paired Student's t test was used to compare the differences between cocultures in the presence or absence of 4-1BBL. P values <0.05 were considered significant.

Results

Culture of leukemic dendritic cells. Culture of AML blasts ($n = 12$) in the presence of cytokines or calcium ionophore resulted in the generation of mature leukemic dendritic cells as evidenced by the significant up-regulation of CD40, CD54, HLA-DR, CD80, CD86, and the dendritic cell maturation marker CD83 (Fig. 1). CD1a was not expressed by the cultured leukemic dendritic cells, in accordance with previous observations (14).

4-1BB and 4-1BBL expression on AML-DC and T cells. 4-1BBL expression was measured on AML-DC. Mean percentage of positives was 2.4% ($n = 10$; SE, 0.9), which seemed to be significantly less compared with 4-1BBL expression on monocyte-derived dendritic cells (mean, 46.2%; SE, 3.4; $n = 3$, $P < 0.05$). 4-1BB expression was measured on AML-DC and T cells from healthy donors and AML patients in complete remission. Mean expression on AML-DC was 1.2% ($n = 10$; SE, 1.1). Unstimulated T cells displayed low mean expression of

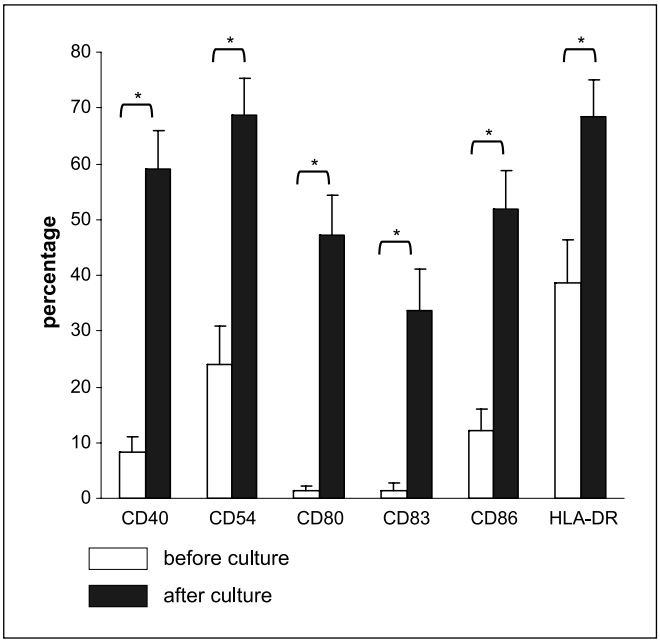

Fig. 1. Immunophenotype of cultured leukemic dendritic cells. AML-DC were cultured as described in Materials and Methods. Immunophenotype of the cells was determined before and at the end of culture. Columns, mean percentages (n = 12); bars, SE. *, $P < 0.01$.

4-1BB with 0.3% positive $CD4^+$ T cells (SE, 0.4%; n = 7) and 1.2% $CD8^+$ T cells (SE, 0.6; n = 7). At day 1 following stimulation, T cells significantly increased the expression of 4-1BB ($CD8^+$ T cells: mean, 40.9%; SE, 4.1; $P < 0.001$; $CD4^+$ T cells: 13.4%; SE, 2.5; $P = 0.01$, n = 7). $CD8^+$ T cells showed a significantly higher 4-1BB expression compared with $CD4^+$ T cells ($P = 0.002$). No differences in 4-1BB expression between T cells derived from healthy donors and T cells derived from AML patients in complete remission were observed (data not shown).

4-1BBL increases T-cell expansion and differentiation in cocultures of leukemic dendritic cells and naïve T cells. Because, apart from T cells, 4-1BB is also expressed by NK cells and B cells, and NK cells and B cells are known to exert effects on T cells, allogeneic cocultures were conducted with isolated naïve T cells from healthy donors. T cells were cocultured with leukemic dendritic cells with or without the addition of 4-1BBL (n = 3). T cells activated by AML-DC in the presence of 4-1BBL showed significantly increased cell numbers of both $CD4^+$ and $CD8^+$ T cells at the end of the coculture compared with cocultures in the absence of 4-1BBL (Fig. 2A). $CD8^+$ T cells displayed the most pronounced increase, indicating that 4-1BBL exerted its effect predominantly on $CD8^+$ T cells.

$CD8^+$ T-cell subpopulations with different activation states were identified based on their expression of CD45RA and CD27. Percentage naïve ($CD45RA^+/CD27^+$) T cells declined during cocultures, whereas the central ($CD45RA^-/CD27^+$) and effector ($CD45RA^-/CD27^-$) memory populations increased significantly in cultures in the presence of 4-1BBL (Fig. 2B). A rapid and transient increase of effector ($CD45RA^+/CD27^-$) T cells was observed upon the addition of 4-1BBL. Literature suggests that 4-1BB targeting predominantly has an effect on $CD8^+$ T cells, but that $CD4^+$ T cells respond to 4-1BB targeting as well (6, 22). Indeed, analysis of $CD4^+$ subpopulations revealed a similar trend with regard to differentiation compared with the $CD8^+$ T-cell population with significantly decreased naïve populations and increased memory and effector $CD4^+$ T-cell populations in cocultures in the presence of 4-1BBL (Fig. 2C). Thus, the addition of 4-1BBL to cocultures of AML-DC and naïve T-cell populations enhances T-cell proliferation predominantly of the $CD8^+$ T-cell population, as well as differentiation of both $CD4^+$ and $CD8^+$ T cells.

To mimic the *in vivo* situation more closely, we next did experiments using the complete lymphocytic population as responder cells (n = 4; data not shown). Similar to cultures with naïve T cells, significantly increased expansion of $CD8^+$ T cells in cocultures with 4-1BBL was observed compared with cultures without 4-1BBL ($P = 0.009$). In contrast, no significant differences between cultures with or without 4-1BBL were detected in terms of $CD4^+$ T-cell expansion. Regarding T-cell differentiation, similar effects of 4-1BBL on the induction of $CD4^+$ and $CD8^+$ effector and memory subpopulations were observed as were shown for cultures with isolated naïve T cells. Percentage naïve $CD4^+$ and $CD8^+$ T cells decreased significantly more in the presence of 4-1BBL ($P = 0.04$ and $P = 0.037$, respectively). Accordingly, significant increased differentiation toward central and effector memory $CD8^+$ T-cell populations ($P = 0.028$ and $P = 0.04$, respectively) and $CD4^+$ memory populations ($P = 0.005$) occurred in presence of 4-1BBL.

WT-1 specificity and TCR Vβ repertoire skewing in 4-1BB–targeted cocultures. Addition of 4-1BBL facilitated sufficient T-cell expansion for subsequent analysis of T-cell specificity. The WT-1 leukemia-associated antigen is overexpressed in the vast majority of AML samples and can be used to establish leukemia-specific reactivity (23, 24). Because WT-1 dextramers are HLA-A2 restricted, experiments done with $HLA\text{-}A2^+$ donor T cells could be analyzed for WT-1 specificity (n = 2). A 35-fold expansion of the WT-1 dextramer–positive $CD8^+$ T cells was detected, already after 14 days of culture, indicating leukemia-specific reactivity (Fig. 3A). In a second coculture, a 75-fold increase of the WT-1 dextramer–positive T cells at the end of coculture was observed (Fig. 3A). Cocultures without 4-1BBL did not expand sufficiently to reliably perform the WT-1 analysis. Additionally, skewing of the TCR Vβ repertoire, indicative of the outgrowth of specific T-cell clones, was studied. Skewing toward Vβ 7.1 and 14 was observed in 40% to 50% of $CD8^+$ T cells primed in the presence of 4-1BBL, constituting a clear overrepresentation (Fig. 3B).

Functional analysis of T cells primed with AML-DC in the presence of 4-1BBL. At the end of cocultures, T cells were evaluated for IFN-γ production, a T helper (Th) 1 cytokine, and IL-4 and IL-10 production, Th2 cytokines. Analysis of cytokine secretion in supernatants showed significantly higher levels of IFN-γ in allogeneic cocultures when 4-1BBL was present, whereas low levels of IL-4 and IL-10 were detected (Fig. 4A). Similarly, significantly increased percentages of IFN-γ–producing T cells were detected in allogeneic cocultures when 4-1BBL was added compared with cocultures without 4-1BBL (before culture: 15%; SE, 0.5; control cultures without 4-1BBL: 22%; SE, 1.0; control cultures with 4-1BBL: 40.3%; SE, 4.0; $P = 0.001$, n = 4), whereas percentages of IL-4–producing T cells remained below 5% in both cocultures, thus pointing toward an overall Th1-skewed cytokine profile.

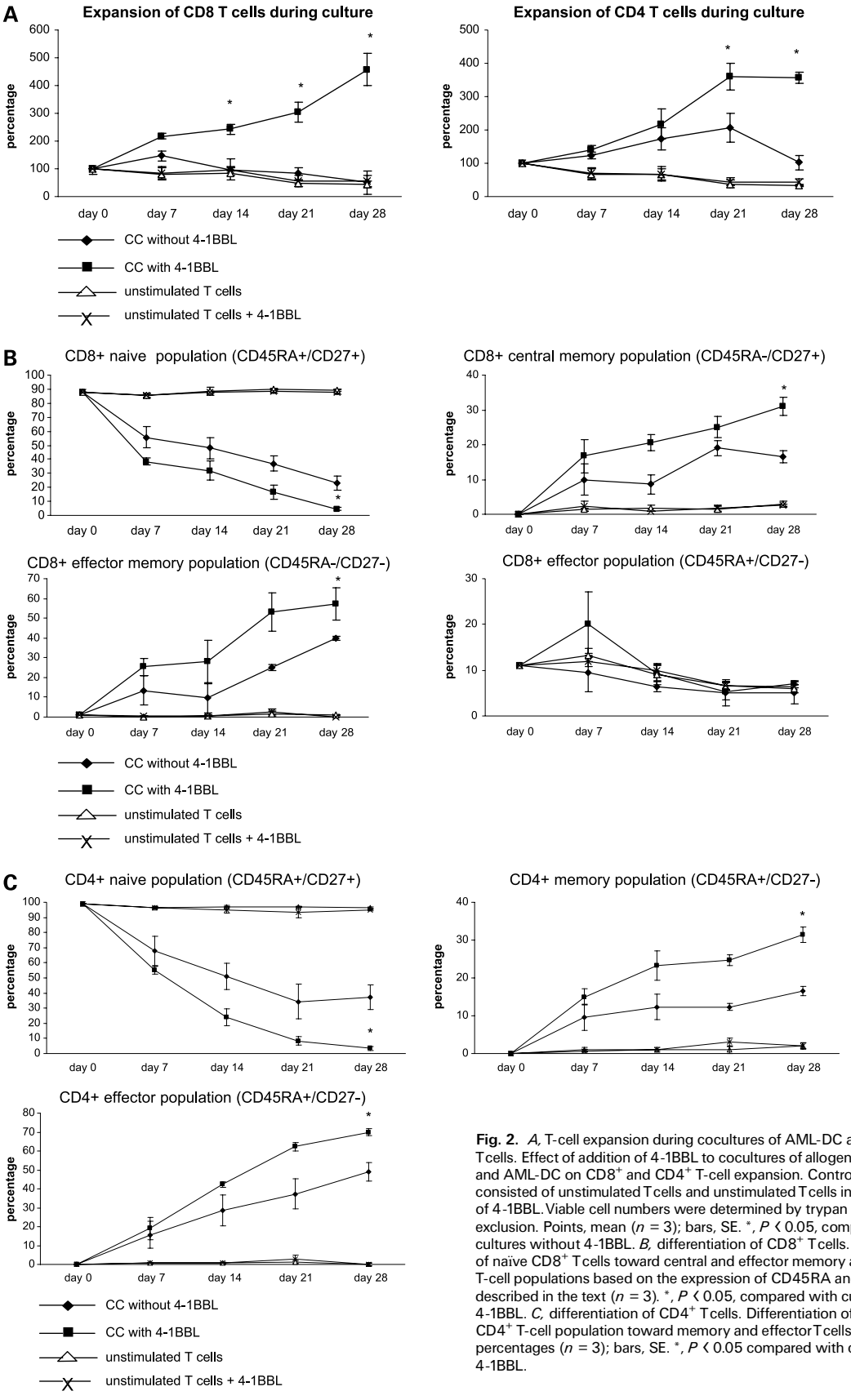

Fig. 2. *A,* T-cell expansion during cocultures of AML-DC and allogeneic T cells. Effect of addition of 4-1BBL to cocultures of allogeneic naïve T cells and AML-DC on $CD8^+$ and $CD4^+$ T-cell expansion. Control cultures (*CC*) consisted of unstimulated T cells and unstimulated T cells in the presence of 4-1BBL. Viable cell numbers were determined by trypan blue dye exclusion. Points, mean ($n = 3$); bars, SE. *, $P < 0.05$, compared with cultures without 4-1BBL. *B,* differentiation of $CD8^+$ T cells. Differentiation of naïve $CD8^+$ T cells toward central and effector memory and effector T-cell populations based on the expression of CD45RA and CD27 as described in the text ($n = 3$). *, $P < 0.05$, compared with cultures without 4-1BBL. *C,* differentiation of $CD4^+$ T cells. Differentiation of the naïve $CD4^+$ T-cell population toward memory and effector T cells. Points, mean percentages ($n = 3$); bars, SE. *, $P < 0.05$ compared with culture without 4-1BBL.

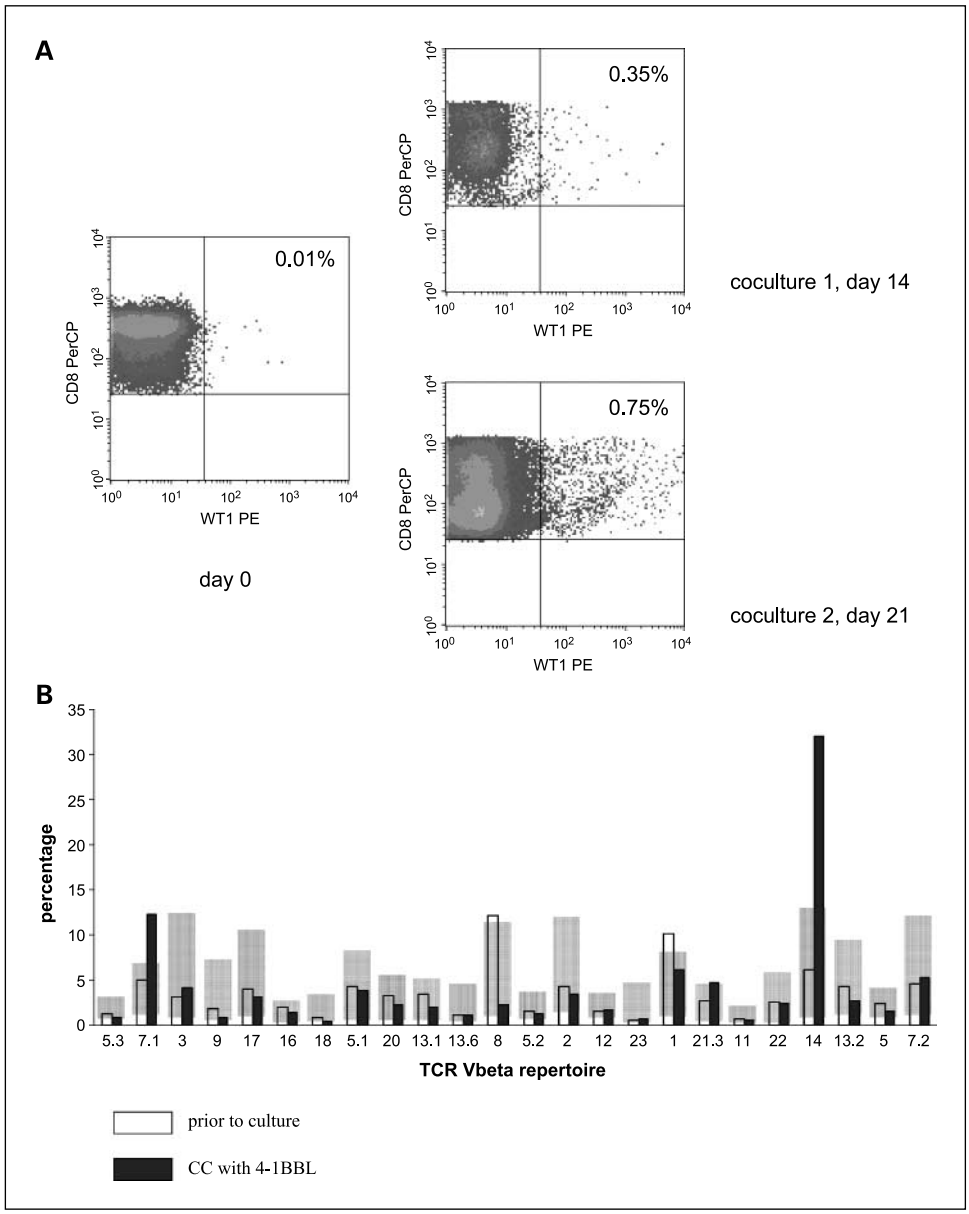

Fig. 3. *A,* WT-1 specificity of allogeneic T cells primed with AML-DC in presence of 4-1BBL. WT-1 specificity of allogeneic naïve T cells cocultured with AML-DC in the presence of 4-1BBL, detected by WT-1 dextramer staining. Two independent cocultures, measured at days 14 and 21, started from the same allogeneic T cells. *B,* TCR Vβ repertoire analysis. Skewing of the TCR Vβ repertoire of allogeneic $CD8^+$ T cells activated by AML-DC in the presence of 4-1BBL. TCR Vβ overrepresentation, assumed to signal clonal $CD8^+$ T-cell expansions, was defined as exceeding 2× the SD of reference values ($SD_{Vb7.1}$, 1.23; SD_{Vb14}, 2.55). One representative experiment. Gray shading, upper and lower reference values.

Cytotoxicity analysis showed that allogeneic whole lymphocyte populations primed with AML-DC in the presence of 4-1BBL showed a markedly increased cytolytic capacity directed against primary leukemic blasts compared with unprimed T cells (Fig. 4B). K562 cells, sensitive for NK cell–mediated killing, were not lysed by these AML-DC–primed lymphocytes, indicating that the observed cytolysis was T cell mediated rather than NK cell mediated (Fig. 4B; refs. 25, 26).

Increased TCR Vβ skewing and MHC-restricted cytolysis in autologous cocultures in presence of 4-1BBL. To examine the feasibility of 4-1BB targeting in an adjuvant therapy setting, autologous T-cell and AML-DC cocultures were done, with a focus on the effects on $CD8^+$ T cells (n = 4). Similar to allogeneic cocultures, increased $CD8^+$ T-cell expansion was observed in the presence of 4-1BBL compared with cocultures without the addition of 4-1BBL (Fig. 5A). Proliferative differences occurred at least 2 weeks later than observed in allogeneic cocultures. Differentiation toward effector and memory subpopulations was detected, but no significant differences between the presence and absence of 4-1BBL were found (data not shown).

TCR Vβ analysis could be assayed in two of four cocultures. As shown by the example in Fig. 5B, skewing in the Vβ repertoire of $CD8^+$ T cells was observed during autologous cocultures in a more pronounced manner in the coculture in the presence of 4-1BBL, indicative of the expansion of specific T-cell clones. In the second coculture, the yield in the culture without 4-1BBL was not sufficient to perform the TCR Vβ analysis. Although no firm conclusions may be drawn from these observations, the second coculture in the presence of 4-1BBL resulted in skewing in the Vβ repertoire of $CD8^+$ T cells, suggesting the expansion of a specific T-cell clone (Vβ 13.2; 27% of total $CD8^+$ T-cell population; SD, 1.80). In addition,

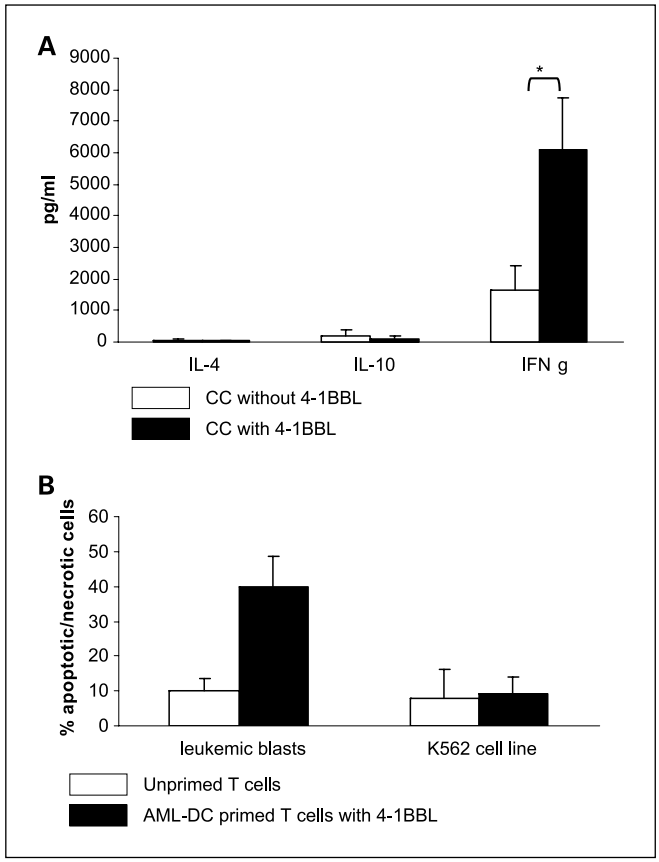

Fig. 4. *A,* T-cell cytokine profile skewing during allogeneic T-cell/AML-DC cocultures. Coculture supernatants were analyzed for IFN-γ, IL-4, and IL-10 secretion by ELISA. Columns, mean of supernatants collected at day 21 ($n = 5$); bars, SE. *, $P < 0.05$. *B,* allogeneic cytotoxicity assay. Cytolytic capacity of T cells primed with AML-DC in the presence of 4-1BBL compared with unprimed T cells. The leukemic cell line K562 was used as a control for NK cell – mediated cytolysis. Percentage killing was corrected for spontaneous killed blasts. T cells in the coculture in the absence of 4-1BBL did not expand sufficiently to perform a cytotoxicity assay. Columns, mean of duplicate measurements (effector/target ratio = 5:1); bars, SE.

AML blast cytolytic capacity of autologous $CD8^+$ T cells primed with AML-DC in the presence of 4-1BBL could be confirmed and was proven to be MHC restricted (Fig. 5C).

Discussion

Although not yet proven in clinical studies, *in vitro* data that we and others have generated show that AML-DC possess all the prerequisite functions needed to elicit an immune response *in vivo* (14, 16, 17). In a phase I pilot study on chronic myelogenous leukemia – derived dendritic cell vaccination in the advanced-stage disease, delayed type hypersensitivity responses representing autologous chronic myelogenous leukemia – specific T-cell responses were detected (27) However, clinical dendritic cell vaccination studies have, until now, shown limited success (18, 19). Targeting costimulatory pathways that are known to prolong T-cell survival and function could be instrumental to potentiate immune responses elicited by tumor-specific dendritic cells (28).

In this study, we found that mature AML-DC lack the expression of 4-1BBL, indicative of impaired costimulatory signaling that might result in suboptimal T-cell responses. We showed that 4-1BBL can be used as a potent adjuvant to enhance leukemia-specific T-cell responses evoked by leukemia-derived dendritic cells. Targeting of 4-1BB by its ligand resulted in an increased proliferation of predominantly the $CD8^+$ T-cell population and an increased induction of the differentiation of both $CD4^+$ and $CD8^+$ T cells, the latter of which was shown capable of exerting MHC-restricted killing of primary leukemic blasts.

4-1BB is expressed not only by activated T cells, but also by dendritic cells, which may deliver costimulatory signals ultimately leading to cytokine secretion, such as IL-12 and IL-6, and improved ability to stimulate T-cell responses (5). However, besides lacking 4-1BBL expression, mature AML-DC also do not express 4-1BB, implying that 4-1BBL did not exert its observed T-cell stimulatory effect via AML-DC.

4-1BB, expressed on activated T cells, provides a costimulatory signal resulting in IL-2 production and up-regulation of antiapoptotic genes, such as *bcl-x_L* and *bfl-1*, thus prolonging survival (29 – 31). Ligation of 4-1BB prevents and even reverses T-cell anergy *in vivo* (32). Additionally, 4-1BB targeting has been shown to enable the rejection of poorly immunogenic tumors (6, 7). CD28 signaling by CD80 and CD86 seems mandatory to elicit these immunogenic responses; thus, a combinatorial approach of T-cell activation by dendritic cells and 4-1BB targeting is preferable (7). In mouse models, the administration of anti-4-1BB agonistic monoclonal antibodies or 4-1BBL proved to increase antitumor reactivity induced by dendritic cell – based vaccines presenting a human tumor – associated antigen, demonstrating its relevance as an adjuvant in the dendritic cell vaccination approach (33, 34).

The increased expansion of $CD8^+$ T cells, in combination with a similar, although weaker, effect on $CD4^+$ T cells shown upon the *in vivo* administration of agonistic anti-4-1BB antibodies, is confirmed in our *in vitro* experiments (6, 22). The observed increased 4-1BB expression of activated $CD8^+$ T cells compared with activated $CD4^+$ T cells is consistent with this.

In accordance with published data, stimulation via 4-1BB, expressed on T cells primed by AML-DC, led to the induction of a significantly larger pool of both effector and central memory T cells as opposed to cocultures without 4-1BBL, whereas little differences were observed in the generation of effector T cells (35 – 37). Autologous cocultures show only slight differences in levels of T-cell differentiation between cocultures with and without 4-1BBL. Because proliferative differences between autologous cocultures with and without 4-1BBL did not occur until the 5th week of coculture, it is also likely that differences in differentiation levels arise at a later time point. Furthermore, because autologous cocultures were conducted with T cells from AML patients in complete remission, differences might reflect the effects on the immune system resulting from high-dose chemotherapy. For example, after stem cell transplantation, $CD8^+$ T cells reappear more rapidly (i.e., within 6 months), compared with $CD4^+$ T cells, which remain at low frequencies even after 1 year (38). Other studies suggest that T cells after exposure to chemotherapy show increased responsiveness upon optimal costimulation that compensates for quantitative defects (39).

It is thought that $CD8^+$ T cells with effector and effector memory phenotype are the cells with the strongest cytolytic

capacity and high releases of IFN-γ. However, it was recently observed that $CD8^+$ central memory T cells were superior in inducing antitumor reactivity compared with the effector memory T-cell subpopulation (40). Although a rapid and transient effect of 4-1BBL addition was found on the generation of effector T cells, generation of central and effector memory populations, capable of exerting effector functions, was significantly improved by the addition of 4-1BBL.

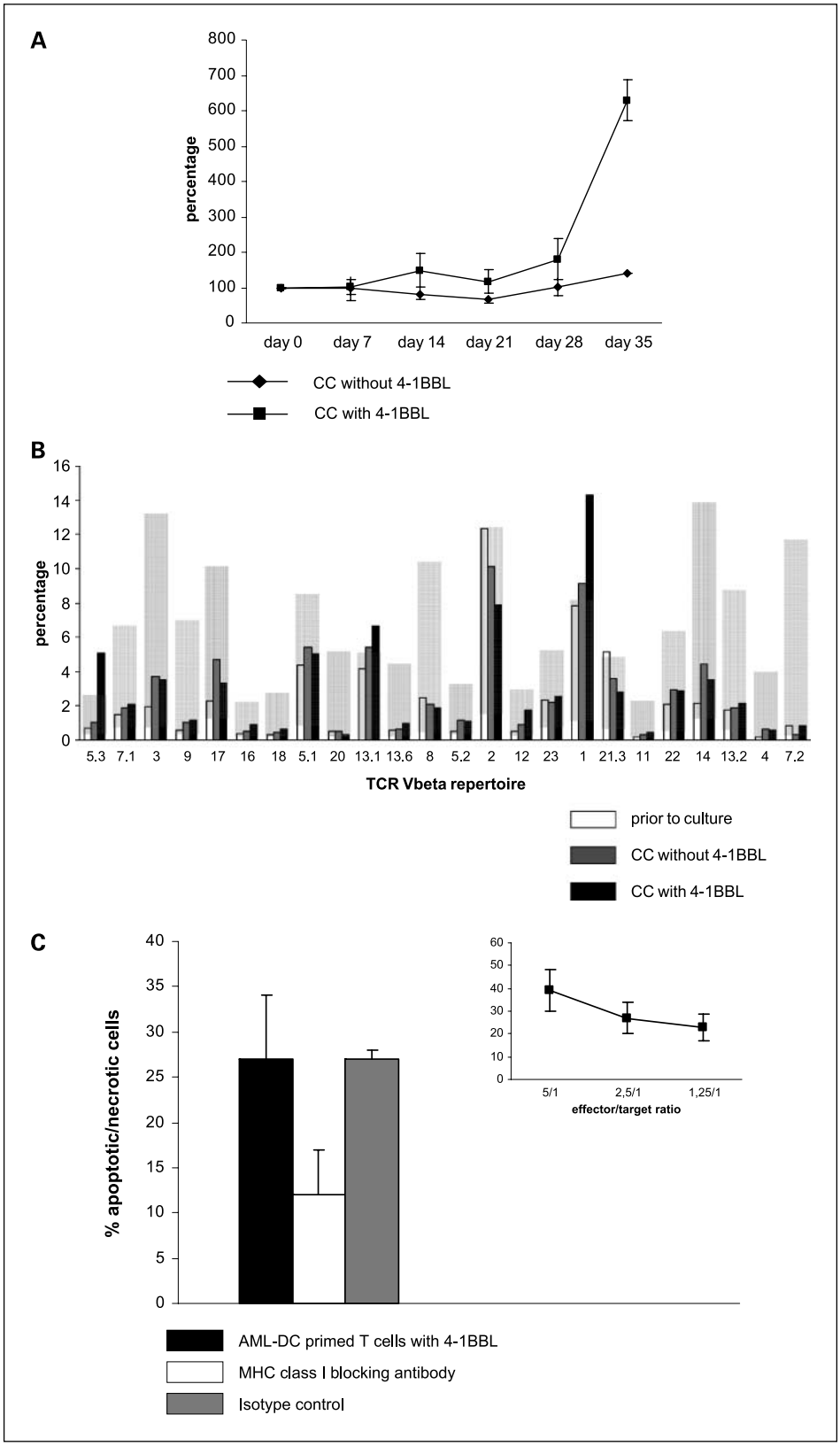

Fig. 5. *A,* $CD8^+$ T-cell expansion during autologous cocultures. Effect on $CD8^+$ T-cell expansion of the addition of 4-1BBL to cocultures of autologous T cells and AML-DC. Control cultures consisted of unstimulated T cells and unstimulated T cells in the presence of 4-1BBL. Viable cell numbers were determined by trypan blue dye exclusion. Points, means ($n = 4$); bars, SE. Due to limited numbers of AML-DC in these patients, two of four cocultures could only be restimulated twice. *, $P < 0.05$, compared with cultures without 4-1BBL. *B,* TCR Vβ repertoire analysis of autologous cocultures of AML-DC and T cells in the presence of 4-1BBL. TCR Vβ overrepresentation or underrepresentation, assumed to signal clonal $CD8^+$ T-cell expansions, was defined as exceeding 2× the SD of reference values (Vβ1, 1.62; Vβ5.3, 0.39). Gray shading, upper and lower reference values. *C,* autologous cytotoxicity assay. MHC-restricted cytolytic capacity of autologous $CD8^+$ T cells primed by AML-DC in the presence of 4-1BBL (effector/target ratio, 2.5:1). Percentage killing was corrected for spontaneously killed blasts. Inset, dose response – related cytotoxicity. Columns, mean of duplicate measurements; bars, SE.

Long-lasting immunity by the generation of memory T cells that execute effector functions upon antigenic restimulation, obtained through priming with AML-DC in the presence of 4-1BBL, is likely to be of great importance to prevent relapses that so frequently occur in AML patients.

NK cells and B cells display the expression of 4-1BB, enabling reciprocal activating interaction between dendritic cells and NK cells (41–44). Because it is known that intense crosstalk exists between T cells and NK cells, as well as T cells and B cells, 4-1BBL could potentially exert its effect on T-cell responses via NK cells or B cells. Although 4-1BB signaling does not improve the cytolytic activity of NK cells, it might promote $CD8^+$ T-cell function via increased cytokine release (45). However, in our study, this seems unlikely because no proliferative or differentiation induction differences between cultures conducted with whole lymphocyte populations and cultures with naïve T cells could be shown. Additionally, observed cytotoxic responses proved to be non–NK cell mediated.

Triggering 4-1BBL significantly increased the production of IFN-γ, whereas IL-4 and IL-10 production remained low, thus effectively enhancing the Th1 response induced by AML-DC. Overrepresentation and underrepresentation of Vβ clones in the T-cell repertoire, indicative of selective outgrowth of $CD8^+$ T-cell clones, were found not only in allogeneic but also in autologous cocultures, resulting in a profound cytolytic capacity directed against primary leukemic blasts of T cells primed by AML-DC in the presence of 4-1BBL. The considerable increase in WT-1–specific T cells during allogeneic cocultures with AML-DC in the presence of 4-1BBL, which were absent at the start of cocultures, provides evidence for the potentiation of a leukemia-specific T-cell response.

To summarize, we showed that 4-1BBL is an effective adjuvant to increase $CD8^+$ T-cell expansion and function evoked by leukemia-derived dendritic cells. We envision an improved clinical outcome of AML-DC vaccination through coadministration of 4-1BBL.

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