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Alloreactivity and anti-tumor activity segregate within two distinct subsets of cytokine-induced killer (CIK) cells: implications for their infusion across major HLA barriers

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Abstract

Donor-derived cytokine-induced killer (CIK) can be infused as adoptive immunotherapy after hematopoietic cell transplant (HCT). Promising results were recently reported in HLA-identical HCT, where mild grafts versus host (GVH) events were observed. To extend this strategy across major HLA barriers (e.g. HLA-haploidentical HCT), further studies on CIK cells' alloreactivity are needed. We hypothesized that alloreactivity and anti-tumor activity of CIK cells segregate within two different cell subsets and could consequently be separated according to CD56 and CD3 expression. We tested CIK cells expanded from seven patients who underwent HCT as treatment of metastatic colorectal cancer. We found that CIK cells maintained their alloreactivity across major HLA barriers when tested as bulk population; after CD56-positive selection, anti-tumor activity was restricted to the CD3+/CD56+ cell fraction and alloreactivity versus HLA-mismatched PBMC was restricted to the CD3+/CD56− cell fraction. Bulk CIK cells from engrafted patients did not exhibit alloreactivity in response to host- or donor-derived PBMC, confirming their low potential for GVH across minor HLA barriers. Moreover, we tested if CIK cells expanded from engrafted patients after HCT were as effective as donor-derived ones and could be considered as an alternative option. The expansion rate and tumor cell killing was comparable to that observed in sibling donors. In conclusion, depletion of CD3+/CD56− cells might reduce the risk of GVH without affecting the tumor-killing capacity and could help extending CIK infusions across major HLA barriers. Engrafted patients after HCT could also be considered as an effective alternative option to donor-derived CIK cells.

Introduction

Cytokine-induced killer (CIK) cells are a subset of T lymphocytes with a NK T cell (NKT) phenotype expressing both the CD3 and the CD56 markers. CIK cells are mostly CD8+, express a heterogeneous TCR repertoire, are CD1d independent and can be reproducibly expanded *in vitro* from bone marrow or PBMC over a 3-week time period (1–4). CIK cells are capable of a broad MHC-unrestricted anti-tumor activity against both syngeneic and allogeneic hematological malignancies, as documented both *in vitro* and *in vivo* by murine models (1, 4, 5).

In pre-clinical studies conducted to date, lymphoma cell lines and fresh leukemic blasts have been the main tumor targets (1, 5, 6). Limited data are available on the efficacy of CIK cells against solid tumors, although efficient killing

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of hepatocarcinoma by conventional CIK and of ovarian carcinoma by CIK redirected against the tumor by a bispecific antibody has been published (7–9). The mechanism of tumor killing by CIK cells is perforin mediated but, to date, it is not completely known which molecules are involved in tumor recognition (10). An important role is played by NKG2D homodimer, which mediates the interaction between CIK cells and tumor targets expressing NKG2D ligands (11).

Two phase I clinical trials reported the successful *ex vivo* expansion and reinfusion of CIK cells from patients with refractory malignant lymphoma and hepatocellular carcinoma without any relevant toxicity and with few (3/22) but significant clinical responses (12, 13).

Allogeneic hematopoietic cell transplantation (HCT) is an established therapy for hematological malignancies (14) and more recently studies of HCT after non-myeloablative conditioning (NMT) have been extended to the field of solid tumors (15–19). Most of the therapeutic effect of NMT depends on an immune response of allogeneic donor T cells to recipient malignant cells, referred to as a graft versus tumor (GVT) effect. NMT has shown to reduce regimen-related toxicities in comparison with conventional myeloablative conditioning but graft-versus-host disease (GVHD) and disease progression remain significant challenges. The beneficial allogeneic GVT effect may be improved by the infusion of donor lymphocytes, even if the risk of severe GVHD remains the major drawback (20–23).

In the allogeneic HCT setting, the GVHD potential of CIK cells was found to be very low in mice models (1, 4, 5). This makes CIK cells an intriguing alternative to bulk donor lymphocytes infusion (DLI), especially when donor and recipient have one or more HLA mismatch. Recently, Introna *et al.* (24) described a very interesting application of donor-derived CIK cells, which were infused in patients with hematological malignancies who relapsed after HCT. They reported 4/11 (36%) cases of acute GVHD (⩽grade II) after the infusion of donor-derived CIK cells, three from HLA-identical siblings and one from HLA-matched unrelated donor, who progressed to extensive chronic GVHD in 2 cases (24). Even if the described GVHD events are mild when CIK cells are infused across minor HLA barriers, the observed alloreactivity raises concerns about their potential use across major HLA barriers (e.g. HLA-haploidentical transplants).

A deeper comprehension of CIK cells' alloreactive potential is needed to improve the safety of their infusion across both minor and major HLA barriers. We hypothesized that the anti-tumor activity and the alloreactivity of CIK cells segregate within two different cell subsets. At the end of CIK cells *ex vivo* expansion, there is constantly a certain amount, usually about 30–40%, of lymphocytes not expressing the CD56 marker. We tested if the CD56− fraction of CIK cells might be responsible for the residual alloreactivity and could be depleted without affecting the overall tumor-killing capacity. In our study, we expanded CIK cells from patients who received an NMT for metastatic colorectal cancer (CRC). This peculiar transplant setting provided the opportunity to further test CIK cells' anti-tumor activity against solid tumors and to explore engrafted patients as an alternative source for efficient and safe generation of CIK cells.

Materials and methods

CIK cultures

Cultures were started with cryopreserved PBMC collected from CRC patients before and after NMT and from their sibling donors. All patients were receiving cyclosporine as immunosuppressive treatment when samples were collected after NMT. In some experiments, fresh collected PBMC from volunteer donors were used. PBMC were cultured overnight in 25-cm^2 cell culture flasks at 2×10^6 ml^{-1} in RPMI (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1000 U ml^{-1} IFN-γ (PeproTech, London, UK) and 10 μg ml^{-1} gentamycin (Gibco). After 18–24 h in culture at 37°C and 5% CO_2, 100 ng ml^{-1} anti-CD3 antibody (PharMingen, San Diego, CA, USA) and 300 U ml^{-1} IL-2 (Chiron Corporation, Emeryville, CA, USA) were added. IL-2 was replaced every 3–4 days and fresh medium added as needed. Phenotypic analysis was performed weekly, and tumoricidal and alloreactive activities were assessed at the end of 3–4 weeks of culture.

CRC patients

Patients from whom CIK cells were expanded in this study had a diagnosis of metastatic CRC and were treated with an NMT from a full HLA-identical sibling donor. All patients were part of a previously published clinical trial (18). Median ages of patients and of their sibling donors at the time of transplant were 57 and 61 (range 36–64 and 34–72), respectively. There were five males and two females in the patient group and four males and three females in the donor group, with a sex mismatch in five out of seven cases. All patients had previously failed at least one chemotherapy line, had a progressive disease at the time of transplant and were receiving cyclosporine as immunosuppression when PBMC samples were collected and cryopreserved (median 55 days after NMT, range 27–85). The median percentage of donor chimerism in CD3+ cells was 55% (range 46–90). All patients signed an informed consent for PBMC collection.

Phenotypic analysis

Samples were stained with FITC, PE, PE–Cyanin 5 (Cy5) or allophycocyanin (APC)-conjugated mouse mAbs and analyzed by flow cytometry using a FACScan instrument (Becton Dickinson, San Jose, CA, USA). The following mAbs were used: CD3–Cy5, CD4–FITC, CD56–PE, CD8–APC or CD8–FITC and CD314–PE (anti-NKG2D, clone 1D11) (all mAbs were from PharMingen). In selected experiments, CIK cells were enriched after expansion by immunomagnetic selection using CD56 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to manufacturer's protocol. The expression of NKG2D ligands MICA and MICB was evaluated by staining tumor targets and PHA-activated PBMC with PE-conjugated MICA-B antibody (PharMingen).

Alloreactivity and cytotoxicity assays

CIK alloreactivity was tested using a [^{3}H]thymidine ([^{3}H]TdR) incorporation assay to measure CIK cells' ability to proliferate in response to allogeneic stimuli. Expanded CIK cells were cultured in a 96-well plate at 1×10^5 cells per well in

200 μl together with γ-irradiated HLA-mismatch PBMC as stimulators (ratio 1:1) for 6 days; [^{3}H]TdR (1 μCi per well, GE Healthcare, Buckinghamshire, UK) was added 18 h before harvesting the cells. Experiments were conducted both on bulk cultures and on positive and negative fractions after CD56 purification. PBMC were irradiated with 30 Gy. To study if a prolonged exposure to allogeneic antigens might increase CIK cells' alloreactivity, the above-described assay was similarly performed, but cultured CIK cells were exposed to allogeneic HLA-mismatched irradiated PBMC for the whole time of the *in vitro* expansion (the ratio of allogeneic PBMC/CIK in culture was 1:1).

In selected experiments, purified CD56+ and CD56− fractions of expanded CIK cells were labeled with 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. CFSE-labeled CIK cells were cultured in a 96-well plate at 1×10^5 cells per well in 200 μl together with γ-irradiated HLA-mismatched PBMC as stimulators (ratio 1:1) for 6 days; IL-2 was present in the culture medium at a concentration of 10 U ml^{-1}. On day 7, cells were stained with anti-CD56, anti-CD8 and anti-CD4 mAbs and the proliferation of each subset was determined by evaluating the logarithmic decrease of CFSE fluorescence intensity. CFSE-labeled CIK cells cultured in the same conditions but without allogeneic stimulators were used as control. Proliferation of CFSE-labeled expanded CIK cells stimulated with high-dose IL-2 (300 U ml^{-1}) was also evaluated.

Cytotoxic activity of expanded CIK cells was assessed using a standard ^{51}Cr release assay. Three CRC cell lines were used as tumor targets: HT29, HCT116 and LS180, which were obtained from the American Type Culture Collection (Manassas, VA, USA). Normal PBMC from HLA-identical or mismatched donors were activated with PHA (2% v/v) and also used as targets (PHA blasts). Target HLA-identical PBMC were obtained either from patients' samples collected before NMT or from their sibling donor. Target human fibroblasts were primary cultures established from skin biopsy of normal individuals. They were maintained in Dulbecco's modified Eagle's MEM (Gibco), containing 10% FBS at 37°C in humidified air with 5% CO_2. Only cells from passage 2–10 were used.

Tumor target cells were labeled with 50 μCi of ^{51}Cr (GE Healthcare) for 2 h at 37°C, washed three times and then incubated overnight (10^4 cells per well) with effector cells at various effector–target ratios in a total volume of 200 μl. The percentage of specific lysis was calculated using the following formula:

$$\text{Experimental} - \text{spontaneous release/maximum} - \text{spontaneous} \times 100.$$

Both cytotoxicity and proliferation assays were performed in triplicate.

Statistical analysis

Median values of CIK expansion were calculated as appropriate. Expansion rates among groups were compared using the Wilcoxon signed rank test. Phenotype among subgroups was compared with the unpaired *t*-test. The mixed model analysis of variance (ANOVA) test was used to compare cytotoxic activity curves of CIK cells among the three groups of engrafted patients, sibling donors and pre-NMT samples. The mixed model ANOVA was also used to compare curves of anti-tumor activity between the CD56+ and the CD56− fractions. Statistical analysis was performed using the software GraphPad Prism 4.

Results

CIK cells expansion

CIK cells were expanded from the PBMC of patients with metastatic CRC collected before (n = 6) and after (n = 7) NMT and from the PBMC of their sibling donors (n = 7). PBMC were incubated on day 0 with IFN-γ and supplemented with anti-CD3 and IL-2 on day +1. With these culture conditions, CD3+/CD56+ cells were successfully expanded over a 3- to 4-week period (Fig. 1). The median increase of CD3+/CD56+ was 45-fold (range, 8–500) in transplanted patients and 38-fold (range, 3–350) in their sibling donors (P = 0.76). When CIK cells were expanded from cryopreserved PBMC collected before transplant, their median expansion rate was 30-fold (range, 7–45) and was comparable to that observed in their corresponding post-transplant samples (P = 0.25).

Phenotype analysis showed that expanded CD3+/CD56+ CIK cells were predominantly CD8+ or CD4−/CD8− (double negative). We did not observe any significant difference in the phenotype of CD3+/CD56+ CIK cells expanded from patients before or after NMT or from their sibling donors. However, a trend toward an increase in the CD4−/CD8− CIK phenotype was observed when CIKs were generated before NMT. Expanded CD3+/CD56− CIK cells had a significantly higher expression of CD4 compared with CD3+/

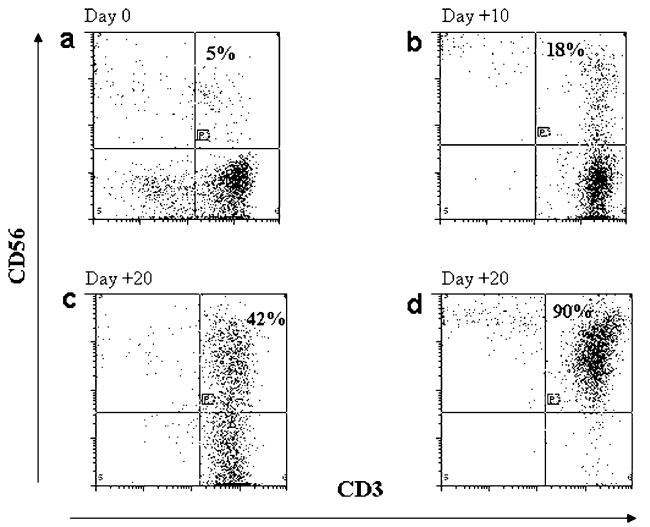

Fig. 1. Phenotype of bulk and purified CIK cultures. (a–c) CD3 and CD56 expression of cultured CIK cells was monitored over time, starting from day 0. (d) Phenotype of CIK cells after CD56-positive selection. Figure shows one representative experiment of CIK cells derived from an engrafted patient after HCT.

Table 1. Phenotype analysis of CD3+/CD56+ and CD3+/CD56− CIK cell sub-populations

Surface markers	% Positive cells, median (range)					
	CD3+/CD56+ cells[a]			CD3+/CD56− cells[a]		
	Patients	Donors	Pre-NMT	Patients	Donors	Pre-NMT
% CD8+	52 (12–78)	75 (20–95)	18 (11–80)	48 (30–49)	32 (20–97)	45 (33–73)
% CD4+	8 (1–19)	2 (0–12)	3 (2–16)	28 (6–61)	25 (1–74)	18 (1–62)
% CD4−CD8−	35 (1–82)	20 (8–73)	66 (4–86)	19 (8–35)	23 (2–49)	25 (11–66)

[a]The percentage of CD8+, CD4+ and CD4−CD8− cells was evaluated within two subsets of CIK cells by gating on CD3+CD56+ cells or on CD3+CD56− cells.

CD56+ cells ($P = 0.0001$), while the expression of CD8 was not significantly different in the two subsets ($P = 0.5$). Data about CIK phenotype are summarized in Table 1. With very few exceptions (one patient before NMT and one donor), NK cells were not significantly expanded after 3–4 weeks of culture, with the median percent value of CD3−/CD56+ cells being 4 (range 2–65), 8 (range 0–19) and 9 (range 2–83) in patients before and after NMT and in their donors, respectively.

CIK cells expanded from patients before and after NMT and from their sibling donors expressed the NKG2D molecule. Expression of NKG2D was significantly higher in CD3+/CD56+ CIK cells compared with CD56− cells ($P < 0.02$) (Table 2).

Taken together, these data show that the expansion rate and the phenotype of CIK cells from engrafted patients are comparable to that of healthy donors. A higher CD4/CD8 ratio is observed on CD56− CIK cells while the CD56+ fraction has a higher expression of the NKG2D molecule.

Tumoricidal activity of bulk and purified CIK cells

The expanded CIK cultures were tested for their anti-tumor activity against colon cancer cell lines and non-malignant targets. CIK cells from engrafted patients efficiently lysed colon cancer target HT29 cells ($n = 7$), LS180 ($n = 4$) and HCT116 ($n = 3$) in a standard ^{51}Cr release assay (Fig. 2A) but not normal fibroblasts ($n = 2$) or activated PBMC ($n = 6$). Tumor killing of CIK cells expanded from engrafted patients was comparable to that of CIK cells expanded from HLA-matched sibling donors ($n = 7$) or from colon cancer patients before transplant ($n = 6$) ($P \geqslant 0.2$). A summary of the tumoricidal activity is reported in Fig. 2(B).

In further experiments, NKT (CD3+/CD56+) were purified by CD56-positive selection from the expanded bulk CIK cell population (up to 95% of purity) (Fig. 1) and their anti-tumor activity was compared with that of the CD56− fraction (Fig. 3). The anti-tumor activity was highly associated with the CD56+ fraction, regardless of whether this cell fraction derived from engrafted patients ($n = 5$) ($P = 0.02$), healthy donors ($n = 5$) ($P = 0.0037$) or patients before transplant ($n = 4$) ($P = 0.026$) (Fig. 3). The presence of pure NK (CD56+/CD3−) in the CD56-purified fraction cells was variable (median 13%, range 2–29%), and the percentage of CD56+/CD3− cells did not correlate with the reported tumor-killing capacity.

Table 2. Expression of NKG2D on CIK cells and of MICA-B on target cells

Cell sub-populations	% NKG2D-positive cells, median (range)		
	Patients	Donors	Pre-NMT
CD3+CD56+	93 (62–98)	97 (86–99)	95 (84–99)
CD3+CD56−	62 (28–87)	54 (34–93)	68 (60–78)
CD3−CD56+	98 (92–100)	99 (95–100)	100 (98–100)
Tumor cell line	% MICA-B-positive cells		
HT29	93		
HCT116	96		
LS180	65		
PHA-activated PBMC	1		

CIK cells' alloreactive potential

The alloreactivity of CIK cells across histocompatibility barriers is an important issue for any possible future application in the transplant setting. We assessed the ability of expanded CIK cells to proliferate against HLA-mismatched targets. Bulk CIK cells could efficiently proliferate in response to allogeneic full HLA-mismatched PBMC. After sorting CD56+ cells from bulk CIK cell cultures, the CD56− fraction fully retained its ability to proliferate in response to allogeneic HLA-mismatched PBMC, while the CD56+ fraction did not show any significant proliferation. Figure 4(A) shows one representative experiment out of three performed with CIK cells generated from engrafted patients and two from healthy sibling donors. In selected experiments, we tested if a prolonged exposure (3 weeks) to HLA-mismatched antigens might enhance CIK cells' alloreactivity. The results overlaid the previous data, purified CD3+/CD56+ cells did not have a significant alloreactive proliferative potential even if previously primed versus allogeneic antigens (Fig. 4B) while maintained their tumor-killing ability (data not shown).

In order to visualize the different contribution of CD4+ and CD8+ cells to the observed proliferation, we labeled both CD56+ and CD56− purified subsets of expanded donor CIK cells with CFSE before stimulating them with irradiated allogeneic PBMC. After 7 days of culture, cells were stained with anti-CD56, anti-CD8 and anti-CD4 and the proliferation of each subset was determined by evaluating the logarithmic decrease of CFSE. We confirmed that CD56− CIK cells have a conserved alloreactive proliferative potential

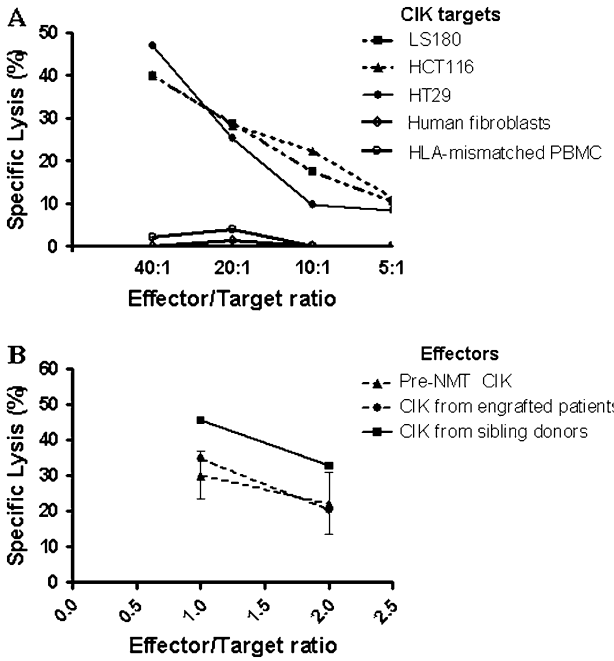

Fig. 2. CIK cells from engrafted patients and sibling donors are equally effective against CRC cell. (A) Cytotoxic activity of CIK cells against human CRC cell lines (LS180, HCT116 and HT29), allogeneic fibroblast and HLA-mismatched activated PBMC (PHA blasts). One representative case of CIK cells derived from an engrafted patient is shown. (B) Cytotoxic activity against the CRC cell line HT29 of CIK cells derived from engrafted patients ($n = 7$), sibling donors ($n = 7$) or CRC patients before NMT ($n = 6$). Figure shows means ± standard errors of the mean of the percent lysis. $P = 0.3$ as determined by two-way ANOVA test.

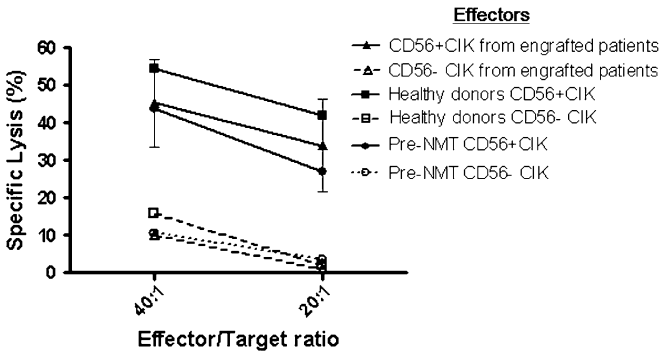

Fig. 3. Anti-tumor activity of CIK cells is restricted to CD56+ subsets. Bulk CIK cell cultures were purified by positive selection according to CD56 expression and the positive and negative fractions were tested against the CRC cell line HT29. Purified cells were obtained from CIK cultures of engrafted patients ($n = 5$), healthy donors ($n = 5$) and of CRC patients before NMT ($n = 4$). Means and standard errors at 40:1 and 20:1 effector–target ratios are represented in the figure. $P < 0.03$ for CD56+ compared with corresponding CD56−, as determined by two-way ANOVA test.

compared with the CD56+ fraction (Fig. 5A and B). Within the proliferating CD56− fraction, CD8+ cells actively contributed to proliferation (Fig. 5C). The CD56+ fraction of expanded CIK cells retained a limited proliferative potential

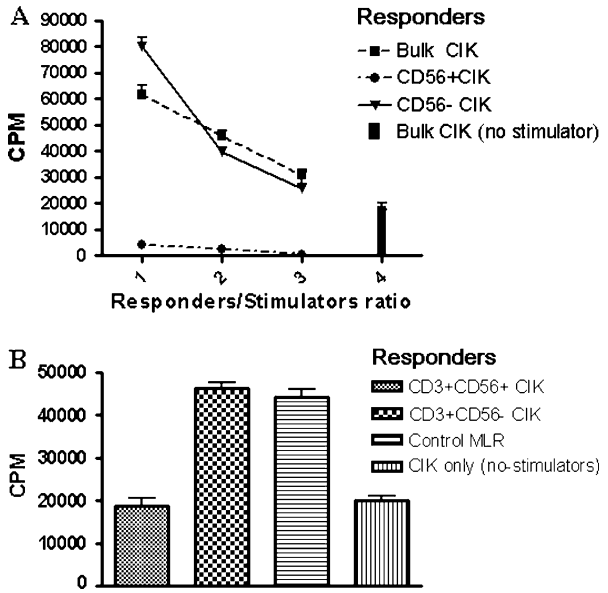

Fig. 4. Alloreactivity across major HLA barriers segregates within two distinct CIK cell subsets. (A) Proliferation in a primary mixed lymphocyte reaction (MLR) of bulk and CD56-purified CIK cells in response to full HLA-mismatched PBMC. Figure shows one representative experiment out of three performed with CIK cells derived from engrafted patients. (B) Similar results were obtained when CIK cells were previously exposed to allogeneic stimulator cells for the whole time of the *in vitro* expansion. A primary MLR was also added as a control, using as responder cells fresh PBMC from the same donor. In all conditions, HLA-mismatched irradiated PBMC were used as stimulator cells (ratio responder/stimulator 1:1). Means and standard errors are represented in the figure.

when properly stimulated with high doses of IL-2 only and without allogeneic PBMC (Fig. 5D).

These data suggest that within bulk CIK cell cultures, alloreactivity and tumor-killing capacity segregate with two different cell sub-populations, respectively, CD3+/CD56− and CD3+/CD56+.

To evaluate the alloreactive potential of CIK cells across minor histocompatibility antigen (mHAg) barriers, we tested the killing activity of CIK cells derived from engrafted patients against normal target cells derived from the host and from their HLA-matched sibling donor. CIK cells could efficiently kill colon cancer cells but did not show a significant cytotoxic activity against host- or donor-derived non-tumor targets such as activated PBMC ($n = 6$) (Fig. 6A). Similarly, bulk CIK cells could not significantly proliferate in response to host- or donor-derived activated PBMC, but they were able to proliferate in response to priming with HLA-mismatched activated PBMC (Fig. 6B).

Discussion

The infusion of donor lymphocytes can improve the allogeneic GVT effect following HCT but the high risk of severe GVHD is an important limitation especially in the presence of major HLA mismatches between donor and recipient. Recently, the infusion of CIK cells has been proposed as an appealing alternative to simple DLI and its feasibility has

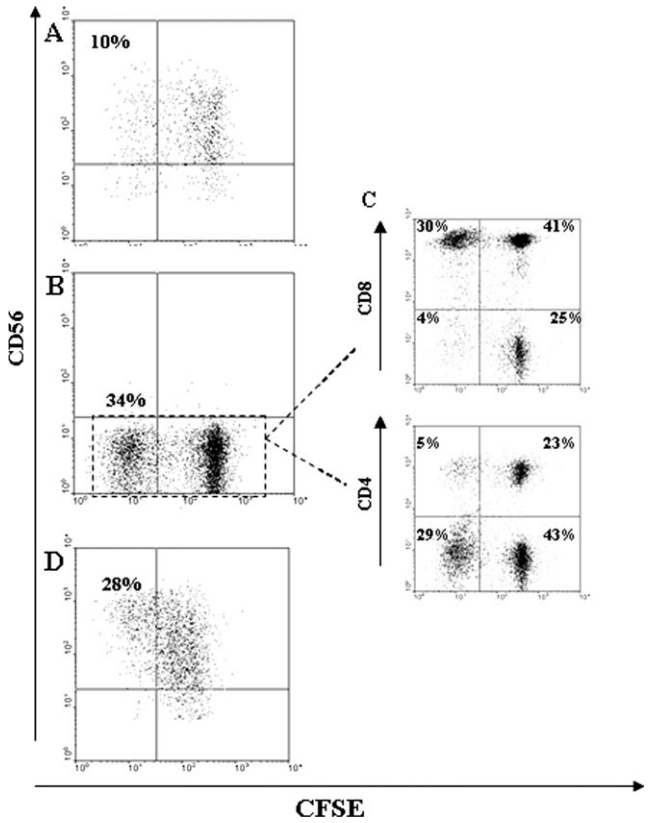

Fig. 5. Proliferation of CFSE-labeled CIK cells. Proliferation in a primary mixed lymphocyte reaction of CD56+ (A) and CD56− (B) CFSE-labeled CIK cells in response to HLA-mismatched PBMC. The different contribution of CD8+ and CD4+ cells to the observed alloreactive proliferation is showed (C). Proliferation of 4-week expanded, CD56-purified CIK cells in response to high-dose IL-2 (300 U ml^{-1}) (D). The reported values of proliferating cells are compared with control CIK cells stimulated with IL-2 only (10 U ml^{-1}). Figure shows one representative experiment out of two performed with CIK cells derived from healthy donors.

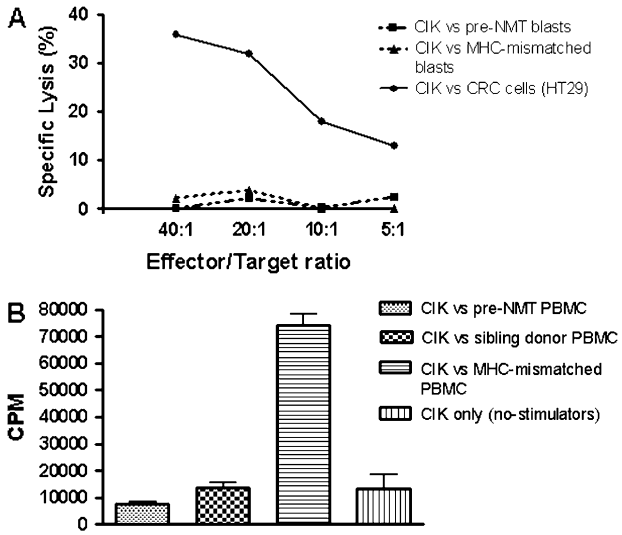

Fig. 6. CIK cells do not have alloreactivity across minor HLA barriers. (A) Spontaneous cytotoxic activity of cultured CIK cells derived from engrafted patients against HLA-matched and mismatched activated PBMC (PHA blasts). HLA-matched blasts were derived from PBMC collected before transplant. CRC cells (HT29) were used as positive control of lytic activity by CIK effectors. (B) Proliferation of the same cultured CIK cells in response to HLA-matched and mismatched PBMC. CIK cells were stimulated with HLA-matched PBMC derived from the sibling donor or from the patient before transplant and with HLA-mismatched PBMC derived from a healthy unrelated donor. Responder/stimulator ratio was 1:1. After 6 days of culture, proliferation was measured with [^{3}H]TdR. Figure shows one representative experiment.

been reported by Introna *et al.* (24). The observed occurrence of mild GVHD suggests a residual alloreactivity of CIK cells and raises concerns about their infusions across major HLA barriers.

In our study, we investigated CIK cells' alloreactivity across major HLA barriers and observed that two cell subpopulations may be functionally and phenotypically distinguished at the end of CIK cells expansion, the first with a fully preserved alloreactive potential while the second just endowed with the anti-tumor capacity. Our findings show that expanded CIK cells are alloreactive across major HLA barriers only if tested as bulk population, while selected CD3+/CD56+ cells showed no or minimal alloreactivity but maintained their tumor-killing potential.

The recent published reports of donor CIK cell infusions after allogeneic HCT only used bulk-expanded cells. Our data suggest that the CD3+/CD56− fraction of CIK cells, retaining their alloreactive potential, could be responsible for GVHD reactions and these findings should be taken into account for future clinical applications especially in the setting of HLA non-identical transplants. Infusion of CD3+/CD56+ selected CIK cells might be a valuable option to reduce the risk of severe GVHD and preserve the GVT effect, opening new possibilities for an adoptive cell therapy after HCT across major HLA barriers.

It could be speculated that a higher CD4/CD8 ratio among CD56− CIK cells or even a general proliferative exhaustion of CD56+ cells might be at the basis of the different alloreactive potential. These observations are probably only partially true. Our data showed in fact that CD8+ cells are the majority even among CD56− cells and we could demonstrate that they are fully capable of alloreactive proliferation. On the other hand, we observed that CD56+ CIK cells still retained a limited proliferative potential when properly stimulated with high dose of IL-2. We think that it is indeed possible that *ex vivo* expanded CD56+ CIK cells are heading toward a generalized exhaustion even if they can still proliferate in response to high-dose IL-2. It is important to underline how this might not be of clinical relevance as long as CD56+ CIK cells retain their full anti-tumor activity.

In our study, we investigated if CIK cells could be expanded from engrafted patients who underwent an NMT for metastatic CRC and where receiving immunosuppressive treatment when PBMC were collected. Current clinical trials are focusing on sibling donors as source of CIK cells for the treatment of hematological malignancies, while the possibility of expanding CIK cells from engrafted patients has been so far unexplored (24–26). Our data show that CIK cells derived from engrafted patients are equally effective and with

a similar expansion rate of those expanded from healthy donors. These findings are in favor of considering the expansion of CIK cells from transplanted patients as a new and valuable option.

With regard to CIK cells' alloreactivity in response to minor HLA antigen mismatches, our *in vitro* model cannot exhaustively reproduce the complex situation of alloreactivity across minor HLA barriers and many issues remain to be addressed. The observation that CIK cells from engrafted patients had no cytotoxic effect against target PBMC collected before transplant suggests a weak alloreactivity across minor HLA barriers, even after a potent *in vitro* stimulation (anti-CD3 and high-dose IL-2). It could be speculated that CIK cells expanded from engrafted patients might be safer than donor-derived ones, because of the tolerance to host mHAg acquired *in vivo* by CIK cell precursors. Further *in vivo* studies are needed to exhaustively investigate this hypothesis and might help translating this approach into a clinical application.

The anti-tumor activity of CIK cells against solid tumors has been thus far relatively less investigated in comparison to that against hematological malignancies. We confirmed CIK cells' ability to efficiently kill CRC cells. In our study, expanded CIK cells could not be tested against HLA-matched or autologous tumor cells, but the observed killing activity against three different CRC cell lines was indicative of an MHC-unrestricted anti-tumor potential, since no or minimal cytotoxicity was observed against normal HLA-mismatched PBMC or fibroblasts.

It has been already elegantly shown that anti-tumor activity of CIK cells occurs through NKG2D signaling and not through the TCR complex (11, 27). We found that NKG2D is highly expressed on expanded CIK cells and that MICA and MICB expression is restricted to tumor target cells, consistently with the experience previously reported in the literature (1, 5, 6, 11). NKG2D has certainly a crucial role in mediating CIK cells cytotoxicity, but it also should be noted that its expression on CD56− cells is fairly abundant (Table 2). It is likely that NKG2D cannot completely account by itself for the significantly higher anti-tumor activity of CD56+ CIK cells compared with the CD56− counterpart, and other molecules might be involved. A possible explanation would be that NKG2D triggering results in different functional outcomes on different cell subsets. For instance, some studies reported that NKG2D ligation induces full activation of NK cells, whereas in T cells it delivers a co-stimulatory signal (28, 29).

In conclusion, CIK cells hold great promises to potentiate the GVT effect after HLA-identical HCT, based on their ability to kill tumor cells in an HLA-independent manner and on their reduced GVHD potential. Our findings suggest that the depletion of the CIK cells sub-population responsible for the residual alloreactivity might further reduce the risk of GVHD, without affecting the tumor-killing capacity, and could help extending this approach across major HLA barriers.

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Abbreviations

ANOVA	analysis of variance
APC	allophycocyanin
CFSE	carboxy-fluorescein diacetate succinimidyl ester
CIK	cytokine-induced killer
CRC	colorectal cancer
Cy5	PE–Cyanin 5
DLI	donor lymphocyte infusion
FBS	fetal bovine serum
GVH	graft versus host
GVHD	graft-versus-host disease
GVT	graft versus tumor
HCT	hematopoietic cell transplant
[^{3}H]TdR	[^{3}H]thymidine
mHAg	minor histocompatibility antigen
NKT	NK T cell
NMT	non-myeloablative transplant

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