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The success of cancer immunotherapy depends on productive tumor cell recognition by killer lymphocytes. γδ T cells are a population of innate-like lymphocytes endowed with strong, MHC-unrestricted cytotoxicity against tumor cells. This notwithstanding, we recently showed that a large proportion of human hematologic tumors is resistant to γδ peripheral blood lymphocytes (PBLs) activated with specific agonists to the highly prevalent Vγ9Vδ2 TCR. Although this probably constitutes an important limitation to current γδ T cell–mediated immunotherapy strategies, we describe here the differentiation of a novel subset of Vδ2⁻Vδ1⁺PBLs expressing natural cytotoxicity receptors (NCRs) that directly mediate killing of leukemia cell lines and chronic lymphocytic leukemia patient neoplastic cells. We show that Vδ1⁺ T cells can be selectively induced to express NKp30, NKp44 and NKp46, through a process that requires functional phosphatidylinositol 3-kinase (PI-3K)/AKT signaling on stimulation with γc cytokines and TCR agonists. The stable expression of NCRs is associated with high levels of granzyme B and enhanced cytotoxicity against lymphoid leukemia cells. Specific gain-of-function and loss-of-function experiments demonstrated that NKp30 makes the most important contribution to TCR-independent leukemia cell recognition. Thus, NKp30⁺ Vδ1⁺ T cells constitute a novel, inducible and specialized killer lymphocyte population with high potential for immunotherapy of human cancer. (Blood. 2011;118(4):992-1001)

Introduction

Tumors develop in hosts endowed with a highly complex immune system that includes various lymphocyte subsets capable of recognizing and destroying transformed cells. It is now widely accepted that, although lymphocytes may constantly patrol tumor formation, cancer cells develop molecular strategies to evade immune surveillance, which are competitively selected under the pressure of the host immune system. This dynamic process, termed “cancer immunoeediting,” is thought to constitute a major obstacle to cancer immunotherapy. Among multiple immune evasion mechanisms, we have recently shown that leukemia and lymphoma primary cells often down-regulate the nonclassic MHC protein, ULBP1, which is critical for recognition of hematologic tumors by γδ T cells expressing the counter-receptor NKG2D. γδ T cells are innate-like lymphocytes that account for 1%-10% of peripheral blood lymphocytes (PBL) of healthy people and are capable of targeting a significant fraction of hematologic tumor cell lines tested in the laboratory. However, we have demonstrated that many lymphoid leukemia cells are resistant to fully activated Vγ9Vδ2 T cells, the dominant subset of γδ PBLs. Furthermore, clinical trials involving the in vivo administration of activators of Vγ9Vδ2 T cells have shown limited success, with objective responses restricted to 10%-33% of patients with either hematologic or solid tumors. Even more modest has been the outcome of trials involving the adoptive transfer of activated and expanded Vδ2⁺ cells, because no objective responses have been reported. In fact, the simple ex vivo expansion of autologous Vδ2⁺ T cells, whose surveillance the tumor managed to escape in vivo, may be condemned to little therapeutic effect on reinjection into the patient. Therefore, we believe it is critical to invest in strategies that endow γδ T cells with additional recognition machinery to detect tumors that have resisted the natural components present in vivo.

Besides Vγ9Vδ2 T cells, Vδ1⁺ T cells are also endowed with potent antitumor cytolytic function, particularly as tissue-associated or tumor-infiltrating lymphocytes. Moreover, Vδ1⁺ T cells can constitute up to 30% of all γδ PBLs and may thus represent an important alternative population for adoptive cell therapy. However, this possibility remains poorly explored.

In this study we identified and characterized a novel Vδ1⁺PBL subset capable of targeting hematologic tumors highly resistant to fully activated Vγ9Vδ2 PBLs. We show that this Vδ1⁺ population owes its specialized killer function to induced expression of natural cytotoxicity receptors (NCRs), which have been mostly regarded as NK-specific markers. Instead, we show that, although neither Vδ1⁺ nor Vδ2⁺ cells express NCRs constitutively, these can be selectively up-regulated in Vδ1⁺ cells by AKT-dependent signals provided synergistically by γc cytokines (IL-2 or IL-15) and TCR stimulation. We further show that NKp30 and NKp44 are both functional in NCR⁺ Vδ1⁺ PBLs, and synergistically contribute to enhanced targeting of lymphocytic leukemia cells, with NKp30 playing the major role in this process. Thus, NKp30⁺ Vδ1⁺ PBL...
constitute a novel promising population for adoptive cell immuno-
therapy of hematologic malignancies.

Methods

Ethics statement

Research involving clinical samples was conducted according to the principles
expressed in the Helsinki Declaration. All procedures were approved by the
review board of Instituto Português de Oncologia de Lisboa (Portugal).

Isolation of human peripheral blood γδ T cells

Peripheral blood was collected from anonymous healthy volunteers, diluted
in a 1:1 ratio (volume-to-volume) with PBS (Invitrogen Gibco), and
centrifuged in Ficol-Paque (Histopaque-1077; Sigma-Aldrich) in a volume
ratio of 1:3 (1 part ficoll to 3 parts diluted blood) for 30 minutes at 1 500 rpm
and 25°C. The interface containing mononuclear cells was collected and
washed (in PBS), and γδ T cells were isolated (to above 95% purity)
by magnetic cell sorting via positive selection (with a FITC-labeled anti-
TCR antibody) or via negative selection (with a cocktail of Biotin-
labeled antibodies; Miltenyi Biotec). When noted, Vδ1+ cells were further
purified by magnetic cell sorting via positive selection with a FITC-labeled
anti-Vδ1 TCR antibody (Fisher Scientific) and anti-FITC microbeads
(Miltenyi Biotec).

Cell culture

Isolated γδ PBLs were cultured at 10^6 cells/mL at 37°C, 5% CO2 in
round-bottom 96 well plates with RPMI 1640 and 2 mM l-glutamine
(Invitrogen Gibco) supplemented with 10% FBS (Invitrogen Gibco), 1 mM
sodium pyruvate (Invitrogen Gibco), and 50 ng/mL of penicillin and
streptomycin (Invitrogen Gibco). The cells were expanded in the presence
of 100 U/mL of rHIL-2 (Roche Applied Science), with or without 10 nM of
HMB-PP (4-hydroxy-3-methyl-but-2-enyl pyrophosphate; Echelon Biosci-
ces) and 1μg/mL of phytohemagglutinin (PHA; Sigma-Aldrich). Cells
were washed and the culture medium was replaced every 5-6 days. To study
the induction of Nkp30 expression, γδ PBLs were cultured in the presence
or absence of 100 U/mL of rHIL-2 (Roche Applied Science), 1μg/mL of
soluble anti-CD3 antibody (eBioscience; clone OKT3), and 20ng/mL of
rHIL-15 (Biologend). For TCR blockade, freshly isolated γδ PBL were
CSFE-labeled and then incubated for 7 days with anti-TCRγδ (Beckman
Coulter; clone IMMU510) diluted 1:20 in complete medium supplemented
with 1μg/mL PHA and 100 U/mL rHIL-2. To study the effects of chemical
inhibitors of signal transduction, the MEK inhibitor U0126 and the PI-3K
inhibitor LY294002 (both from Calbiochem) were added at 10 μM for
a 2-hour incubation period and then maintained in culture with 100 U/mL
rHIL-2 and 1μg/mL PHA for 7 days.

Flow cytometric cell sorting

For sorting of γδ PBL based on the expression of Nkp30 and Vδ1+ TCR,
cells from PHA and IL-2–activated cultures were stained with anti-NKP30
(Biologend; clone P30-15), anti-Vδ1 (Thermo Fisher Scientific; clone
TS8.2), and sorted on a FACSAria cell sorter (BD Biosciences).

Leukemia patient samples

B-cell chronic lymphocytic leukemia cells were obtained from the periph-
ernal blood of patients at presentation, after informed consent and institu-
tional review board approval (Instituto Português de Oncologia de Lisboa,
Portugal). Samples were enriched by density centrifugation over Ficol-
Paque and then washed twice in 10% RPMI 1640.

In vitro tumor-killing assays

All tumor cell lines (details provided in supplemental Table 1, available on
the Blood Web site; see the Supplemental Materials link at the top of the
online article) were cultured in complete 10% RPMI 1640, maintained at
10^5 up to 10^6 cells/mL by dilution and splitting in a 1:3 ratio every 3-4 days.
For cytotoxicity assays, magnetically purified γδ PBL were preactivated for
7-19 days in the presence of IL-2 (100 U/mL) and either 1μg/mL PHA or 10
nM HMB-PP. For receptor blocking, γδ PBLs were incubated for 2 hours
with the blocking antibodies anti-NKp30 (clone F252), anti-NKp44 (clone
KS38), anti-NKp46 (clone KL247), anti-TCRγδ (Beckman Coulter, clones
IMMU510 or B1.1), or anti-Vδ1 TCR (Fisher Scientific, clones TCS1 or
TS8.2). The blocking antibodies were maintained in the culture medium
during the killing assays. Tumor cell lines or leukemia primary samples
were stained with CellTrace Far Red DDAO-SE (1 μM; Molecular Probes,
Invitrogen) and each batch of 3 × 10^5 γδ T cells in RPMI for 3 hours at 37°C
and 5% CO2 on a round-bottom plate with 96 wells. Cells were then stained with annexin
V–FITC (BD Biosciences) and analyzed by flow cytometry. For the
redirected killing assays, PHA and IL-2–activated γδ PBL were incubated for
4 hours with the NCR agonists anti-NKp30 (clone AZ20), anti-NKp44
(clone Z231) or anti-NKp46 (clone Bab281) during a standard 15Cr release
assay.

Flow cytometry analysis

Cells were labeled with the following fluorescent monoclonal antibodies:
anti-CD3–PerCP-Cy5.5 (eBioscience; clone OKT3); anti-TCRγδ–FITC
(eBioscience; clone B1.1); anti-CD69–PE (BD Pharmingen; clone FN50);
anti-NKG2D–PE/Cy7 (Biologend; clone 1D11); anti-2B4–APC (Biolog-
end, clone C17); anti–DNAM-1–Alexa-Fluor647 (Biologend; clone DX11);
anti-NKp30–APC (Biologend; clone P30-15); anti-Vδ2 TCR–PE (Biolog-
end; clone B6); anti-NKp44–APC (Biologend; clone P44-8); anti-NKp46–
AlexaFluor647 (Biologend; clone 9E2); anti-Vδ1 TCR–FITC (Thermo
Fisher Scientific; clone TS8.2); anti-NKp30–PE (Biologend; clone P30-15);
anti-Mouse IgG1–APC Isotype Ctrl (Biologend; clone MOPC-21); anti–
Mouse IgG1–PE Isotype Ctrl (Biologend; clone MOPC-21); anti-CD27–
APC/Cy7 (Biologend; clone O323); and anti-CD56–APC (Biologend, clone
HCDC56). Cell proliferation was measured by following a standard CFSE
staining protocol (CellTrace CFSE Cell Proliferation Kit, Invitrogen; final
concentration 0.5 μM), while apoptosis was assessed by annexin V–FITC
(BD Pharmingen) staining. Cells were analyzed on a FACScanto flow
cytometer (BD Biosciences).

RNA isolation and cDNA production

Total RNA was extracted using the RNeasy Mini Kit according to the
manufacturer’s protocol (QIAGEN). Concentration and purity was deter-
mined by spectrophotometry and integrity was confirmed using an Agilent
2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies).
Total RNA was reverse-transcribed into cDNA using random hexamers and
Superscript II first strand synthesis reagents (Invitrogen).

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed on ABI Prism 7500
FAST Sequence Detection System using SYBR Green detection system
(both from Applied Biosystems). Primers were designed using Primer3
v.0.4.0 online program (http://primer3.sourceforge.net). For each transcript,
quantification was done using the calibration curve method. β2-microglobulin
(B2M), Glucuronidase β (GUSB) and proteasome subunit β type 6 (PSMB6)
were used as housekeeping controls for normalization of gene expression.
The following primers were used: B2M, forward CTAT CGAG
CTGA CTCC AAAG ATAC TTCT CRTC; reverse CTTG CTGG AAGA CAGT
ATCT AATG; PSMB6, forward GGGG GACC CAGA GATT AAAA, reverse
AAAC TGCA CGGC CATG ATA; GUSB, forward GGGG GACC CAGA
TCTC TTG, reverse CTTG CTGA AAGA CAAG TCTG GTAA TCA; B7H6,
forward TCAC CAAG AGGC ATTG CGAC CT, reverse ACCA CTCCT
ACAT CGGT ACTC TC; NKp44, forward CGGT CAGA TTTT ATCT
GCTG GT, reverse CACA CAGG TCTG GCTG; NKp46, forward
AAAC CCCG ACCC TTCC TGA, reverse TGCT GGCT CGCT CTT
AGT; GZMB, forward GGGG GACC CAGA GATT AAAA, reverse
CCAT TTGT CGCT CCAT AGGA G. All samples were run in triplicate and
repeated 3 times. Analysis of the qPCR results was performed using the ABI
SDS v1.1 sequence analysis software (Applied Biosystems).
Statistical analysis.

Differences between subpopulations were assessed using the Student t test and are indicated when significant as *P < .05; **P < .01; and ***P < .001 in the figures.

Results

Enhanced cytotoxicity of γδ PBL cultures activated with pan–T-cell mitogen

We compared the antitumor killing capacity of γδ PBL cultures (always maintained in the presence of IL-2) activated either with PHA, a plant lectin that acts as a potent T-cell mitogen,11 or the specific Vγ9Vδ2 TCR agonist HMB-PP.12,13 Although both regimens were similarly efficient at activating γδ PBLs, as evaluated by CD69 up-regulation and cell proliferation (Figure 1A), we noted that samples activated with PHA were consistently better killers of hematopoietic tumor cell lines than samples (of the same donor origin) stimulated with HMB-PP (Figure 1B-C). This was valid across all donors tested (Figure 1B, supplemental Table 2, and data not shown) and was associated with higher expression of GZMB (Figure 1D), a key component of the lymphocyte cytolytic machinery. Of note, freshly isolated γδ PBLs, which lack GZMB expression (Figure 1D), displayed very poor antileukemia cytotoxicity (<10% killing; not shown), as previously reported.13

Figure 1. Enhanced antileukemia cytotoxicity of γδ PBL cultures activated with pan–T-cell mitogen. (A) γδ peripheral blood lymphocytes (γδ PBLs) were MACS-sorted from the peripheral blood of healthy volunteers (left panel), and stimulated with either HMB-PP and IL-2 or PHA and IL-2 for 4 to 19 days. Activation was evaluated by flow cytometry for CD69 up-regulation (middle panels; levels in freshly isolated control cells are shaded), and total cell numbers are shown on the right panel. (B-C) Preactivated (for 14 days, as in panel A) γδ PBLs were coincubated with DDAOse-labeled leukemia cells for 3 hours. Tumor cell lysis was evaluated by annexin-V staining using flow cytometry. (B) Representative results of 6 different donors for the Bv173 leukemia cell line. Percentages refer to annexin-V+ tumor cells. Basal tumor cell apoptosis (in the absence of γδ PBL) was < 5%. (C) Summary of the results of 6 different donors with 4 leukemia target cell lines. Error bars represent SD (n = 6, *P < .05, **P < .01). (D) Real-time PCR quantification of GzmB mRNA levels in freshly isolated, HMB-PP and IL-2–activated and PHA and IL-2–activated γδ PBL. Data in this figure are representative of 2 to 3 independent experiments with similar results.
The superior cytotoxic function of PHA-stimulated γδ PBL cultures was a surprising finding, because we and others have shown that HMB-PP is a very potent activator of the highly dominant Vγ9Vδ2 PBL subset.13 We were particularly interested that, compared with HMB-PP–activated γδ PBL, PHA-stimulated cultures displayed improved cytotoxicity against various resistant leukemia cell lines, such as Bv-173, REH or HPB-ALL (Figure 1B-C), which we had shown to lack expression of the critical NKG2D ligand ULBP1.2,3 Of note, PHA-stimulated γδ T cells did not target normal (healthy) PBMC (supplemental Figure 1). These data demonstrate that the pan–T-cell mitogen PHA is capable of increasing the cytolytic potential of medium-term (1-3 weeks) γδ PBL cultures against leukemia cells, which could be of great value for adoptive cell immunotherapy.

**Induction of NCR expression on γδ PBLs activated with pan–T-cell mitogen**

We next investigated the mechanism(s) underlying the enhanced cytotoxicity of PHA-activated γδ PBL cultures. We considered that this could be explained by differential expression of receptors such as NKG2D,2,14,15 DNAM-1,16,17 or 2B4,18 all previously shown to participate in tumor cell recognition by killer lymphocytes. However, none of these candidates was differentially expressed between PHA-activated and HMB-PP-activated γδ PBL cultures (Figure 2A). By contrast, and unexpectedly, the natural cytotoxicity receptor Nkp30, an important trigger of NK cell cytotoxicity,19 was specifically found on PHA-stimulated γδ PBLs (Figure 2B; supplemental Figure 2A-B). Furthermore, the other NCR family members, Nkp44 and Nkp46, were also selectively expressed in these samples (Figure 2C-D; see next paragraph).

The proportion of Nkp30+ cells increased steadily with culture time (Figure 2E), suggesting an association of Nkp30 induction with cell proliferation. Although unlikely because of the very low background in fresh samples (Figure 2E), it was possible that a minute subset constitutively expressing Nkp30 could be preferentially expanded in PHA-stimulated γδ PBL cultures. However, experiments with highly (>99%) FACS-purified Nkp30+ cells demonstrated that Nkp30- cells were able to acquire Nkp30 expression as efficiently as unsorted cells on PHA and IL-2 stimulation (supplemental Figure 3). Moreover, under such conditions, Nkp30- and Nkp30+ cells proliferated to similar extent (not shown), further arguing against preferential expansion of Nkp30+ cells under such conditions. These results suggest that Nkp30 expression is induced de novo on γδ PBL activation by PHA and IL-2 treatment, which is coupled to cell proliferation.

**NCRs are selectively expressed by proliferating Vδ1+ T cells**

Considering that HMB-PP had been shown to be an optimal agonist of Vγ9Vδ2 cells,12,13 we hypothesized that our findings derived from PHA-mediated activation of a distinct γδ PBL subset. Consistent with this, we observed that, by contrast with HMB-PP treatment with PHA preferentially expanded Vδ2- cells among γδ PBL (Figure 3A). We verified that this was not because of differences in Vδ2+ cell apoptosis in the 2 experimental conditions (supplemental Figure 2C). The most likely Vδ2+ population to expand so markedly (Figure 3A) were Vδ1+ cells, because other subsets are very rare in the peripheral blood of healthy adults.20 When Vδ1 versus Vδ2 TCR usage was assessed, a dramatic Vδ1+ cell enrichment was found in PHA-activated cultures (>80% of all γδ T cells after 19 days; Figure 3B; supplemental Figure 4).
Conversely, and as described, HMB-PP–activated cultures were progressively dominated by V62+ cells (Figure 3A; supplemental Figure 4).

The induction of NKp30 expression was examined in parallel cultures of isolated V61+ or V62+ cells, which were stimulated with PHA and IL-2. Although neither freshly isolated V61+ nor V62+ cells expressed NKp30 (supplemental Figure 2B), this NCR was strongly induced (on PHA and IL-2 treatment) in V61+ but not V62+ cells (Figure 3C). Moreover, by following CFSE dilution, we demonstrated a striking accumulation of NKp30+ cells with progressive division of V61+ cells (Figure 3C). These data suggest that activation of V61+ cells in PHA and IL-2 cultures induces NKp30 expression concomitantly with cell proliferation.

Whereas high percentages (> 50%) of NKp30+ cells were usually detected after 2 to 3 weeks in culture, NKp44 (~ 30%) and NKp46 (< 20%) were expressed in lower proportions of V61+ cells (Figure 3D). Furthermore, most of NKp44+ or NKp46+ V61+ cells also expressed NKp30 (Figure 3D). We therefore considered NKp30 as the most informative marker of the inducible NCR+ V61+ subset, and we set out to further characterize its differentiation.

**NKp30 induction requires AKT-dependent γc cytokine and TCR signals**

We next dissected the specific signals required for the differentiation of NCR+ V61+ T cells. First, the 2 components of the activation protocol, IL-2 and PHA, were dissociated. IL-2, or its related γc cytokine, IL-15, alone were sufficient to induce some NKp30 expression, but the effect was modest compared with PHA and IL-2 (or PHA and IL-15) combinations (Figure 4A and not shown). On the other hand, PHA alone was not able to keep the cultures viable (data not shown), consistent with the critical role of γc cytokines in the survival of γδ T cells, particularly on activation and proliferation.

Although PHA has been a widely used T cell mitogen, it is also a nonphysiologic compound capable of cross-linking a series of surface receptors, including the TCR. We hypothesized that the molecular mediator of PHA stimulation could be the V61+ TCR complex. We therefore compared the ability of PHA and the OKT3 mAb, which specifically cross-links CD3ε chains of the TCR complex, to induce NKp30 expression (when combined with IL-2 or IL-15) in V61+ T cells. OKT3 was fully capable of mimicking PHA in these assays (Figure 4A-B), thus inducing NKp30 in proliferating V61+ T cells (supplemental Figure 5). Moreover, TCR-γδ blockade in PHA and IL-2 cultures prevented NKp30 induction (Figure 4C). These data suggest that PHA treatment provides TCR signals to induce NCR expression on V61+ PBL. Moreover, the differences between cytokine alone or combination treatments with OKT3 (or PHA) highlight a marked synergy between γc cytokine and TCR signals in this process (Figure 4A-B).
To further explore the molecular mechanisms of NCR induction, we used chemical inhibitors of key signal transduction pathways downstream of γc cytokine receptors and/or TCR signaling. Although blocking JAK signaling triggered extensive cell death before any NCR induction (not shown), coinubcation with the PI-3K/AKT inhibitor LY294002 specifically prevented NKp30 induction in proliferating V61+ T cells (Figure 4D). AKT is involved in transducing both γc cytokine and TCR signals, including TCRγδ signals.13 By contrast, the MAPK/Erk inhibitor UO126 had no detectable effect on NKp30 induction in proliferating V61+ T cells (Figure 4D). Importantly, the selective effect of LY294002 dissociated NCR induction from cell proliferation, thus demonstrating that V61+ T-cell proliferation is necessary (Figure 3C; supplemental Figure 5) but not sufficient (Figure 4D) to induce NKp30 expression. Collectively, these data demonstrate that AKT-dependent γc cytokine and TCR signals synergize to induce NKp30 expression in V61+ T cells.

**Functional NKp30 and NKp44 trigger tumor cell killing by V61+ PBLs**

Although the previous data established clear associations between NKp30 expression and increased cytotoxicity of γδ (V61+) PBL cultures, the functional role of NCRs in this system remained to be formally demonstrated. We therefore undertook gain-of-function and loss-of-function experiments to evaluate the effect of NCR modulation on V61+ enriched (> 80%; supplemental Figure 3A) PBL cultures, which expressed NCRs at levels similar to those in Figure 3D (not shown). First, using a reverse Ab-dependent cytotoxicity assay, we showed that cross-linking of NKp30 or NKp44, but not NKp46, produced significant increases in lysis of the P815 tumor cell targets (Figure 5A). These data demonstrate that induced NKp30 and NKp44 are functional and mediate tumor cell killing. To assess if they played nonredundant roles in targeting leukemia cells, we performed receptor blockade experiments using NCR-specific mAbs (kindly provided by Dr A. Moretta, University of Genova, Italy). We observed significant reductions in tumor cell lysis on NKp30 and NKp44 blockade (Figure 5B). As expected from the results in Figure 5A, NKp46 blockade did not affect tumor cell killing. Interestingly, a synergistic effect between NKp30 and NKp44 was also clearly observed. Of note, TCRγδ blockade in any setting (alone or in combination with anti-NCR mAbs) was a neutral event during the killing assay (Figure 5B). To further establish the TCR-independence of NCR+ V61+ PBL cytotoxicity, we isolated PHA-activated V61+ cells to very high purity (supplemental Figure 6) and used 3 different well-described anti-TCR blocking antibodies, including one (TCS1) specific for the V61+ TCR. Again, we observed no effect on leukemia cell killing (Figure 5C). By contrast, inhibition of NKG2D had a significant (Figure 5C) and dose-dependent (supplemental Figure 6C) impact on tumor lysis. In fact, when we combined NCR and NKG2D inhibition, NCR+ V61+ PBLs could not kill above background levels (Figure 5C). These data suggest that leukemia cell targeting by NCR+ V61+ PBLs is a TCR-independent event mostly mediated by the synergistic function of NKp30, NKp44 and NKG2D.

**NKp30+ V61+ PBLs are specialized killers that target resistant primary lymphocytic leukemias**

To fully characterize the antitumor potential of NCR+ V61+ PBL, we used FACS to sort NKp30+ cells to a high degree of purity (> 99%; Figure 6A) and performed a series of functional assays. As expected (Figure 3D), sorted NKp30+ cells also expressed NKp44 and NKp46 (Figure 6B), and the 3 NCRs were largely stable on the surface of the purified cells when cultured for 2 weeks with IL-2 alone (Figure 6C). These data demonstrate the feasible expansion of a stable NCR+ V61+ T cell subset.

When the cytotoxic function of NKp30+ cells was assessed, an increased targeting of the resistant leukemia cell line Bv173 (among others; not shown) was observed (in comparison with NKp30− counterparts; Figure 6D). This correlated with higher expression of granzyme B (Figure 6E). Moreover, NKp30 expression also associated with higher degree of CD56 expression (supplemental Figure 7), which has been previously linked to cytotoxicity of human lymphocytes, including V62+ T cells.25

Finally, we performed functional killing assays with primary samples obtained from B-cell chronic lymphocytic leukemia patients. We previously showed that such specimens are considerably resistant to γδ PBL activated and expanded with the specific Vγ9V82 TCR agonist HMB-PP,6 a finding confirmed in this study (Figure 6F-G). Importantly, HMB-PP and IL-2–activated γδ PBLs do not express NCRs (Figure 2B). We therefore compared their antitumor cytolytic activity with that of NKp30+ cells isolated from γδ PBL cultures activated with PHA and IL-2. We observed
that NKp30+ γδ PBLs, obtained from 6 different donors, were consistently more efficient at eliminating primary B-CLL cells (Figure 6F-G). These data collectively suggest that highly cytotoxic NKp30+ V61+ PBL are promising new candidates for adoptive cell immunotherapy of hematologic malignancies.

Discussion

Natural cytotoxicity receptors were identified by A. Moretta and colleagues over a decade ago, and were shown to play critical synergistic roles in the antitumor functions of NK cells. In fact, NKp30 and NKp46 are widely considered to be 2 of the most specific NK markers. We now show that the combination of cytokine (IL-2 or IL-15) and mitogenic (PHA or OKT3) stimuli induces NCR expression in a sizeable V61+ PBL subset that is endowed with increased cytolytic activity against hematologic tumors. Although PHA is a nonphysiologic T-cell mitogen, we demonstrated that its effect on NCR induction was fully mimicked by crosslinking the TCR-CD3 complex on V61+ PBL. Thus, NCR induction is coupled to TCR-mediated proliferation of V61+ cells, while also requiring γc cytokine signals. This is consistent with previous reports demonstrating that the in vitro acquisition of NK receptors by liver or umbilical cord T cells depends on IL-15.

Among inducible NCRs, NKp30 is clearly the most important for the antitumor activity of V61+ T cells, based on the proportion of cells that express it (Figure 3D), the higher enhancement in V61+ T-cell cytotoxicity on NKp30 triggering (Figure 5A), and the significant reduction in leukemia cell killing on NKp30 blockade (Figure 5B). This notwithstanding, NKp44 (but not NKp46) is also functional in NCR+ V61+ cells (Figure 5A), and appears to synergize with NKp30 for enhanced tumor targeting (Figure 5B). Of note, NKp30 engagement also augments the production of the key antitumor cytokine, interferon-γ, by NCR+ V61+ cells (supplemental Figure 8).

Both NKp30 and NKp44 have been implicated in human NK cell recognition of virus-infected cells. Regarding tumors, antibody-mediated blocking experiments demonstrated important roles for these receptors in myeloma and melanoma cell targeting. Moreover, lack of NCR expression has been clinically correlated with poor survival in AML patients.

Interestingly, NKp30 and NKp44 are not encoded in the genome of murine strains (such as C57Bl/6 or Balb/c) widely used for laboratory experimentation. On the other hand, the major Vγ9Vδ2 subset of human PBL, and its reactivity toward phosphoantigens, are also primate-specific. These observations highlight the special functional characteristics of primate γδ T cells; however, they also preclude the direct in vivo study of Vγ9Vδ2 or NKp30+ V61+ T cells in the mouse.

Another aspect that currently limits our understanding of NCR function in immunity is the poor definition of their physiologic ligands, most notably in the context of tumors. In fact, viral hemagglutinin was until recently the only well-established ligand for NKp44 and NKp46. However, we obtained no evidence for a role of B7-H6 in NCR+ γδ T cells; however, they also preclude the direct in vivo study of Vγ9Vδ2 or NKp30+ V61+ T-cell targets. Thus, future lines of research should clarify the repertoire of relevant ligands expressed by tumors susceptible to NKp30-mediated cytotoxicity. Moreover, although we have thus far concentrated on hematologic malignancies, upcoming work will address whether NKp30+ V61+ lymphocytes also possess enhanced cytotoxicity against solid tumors. Of note, V61+ T cells have previously been shown to be cytolytic against melanoma and various carcinomas.

V61+ T cells are the predominant γδ T-cell subset during the fetal stage and early life, when they are already able to respond to viral infection. In adults, V61+ T-cell expansions have been associated with CMV infection, HIV-1 infection, and tumors of either epithelial or hematopoietic origin. An attractive prospect for adoptive transfer of activated V61+ T cells is that they may display particularly good capacity for homing to tissues because, contrary to their circulating V62+ counterparts, V61+ cells are preferentially tissue-associated lymphocytes. Interestingly, the
abundance of V\textsuperscript{61+} T cells at mucosal surfaces has been attributed to IL-15, which induces chromatin modifications that control TCR gene rearrangement.\textsuperscript{46}

Our key demonstration that NKp30\textsuperscript{+} V\textsuperscript{61+} cells are capable of targeting primary lymphoid leukemic cells is particularly relevant when taking into account that V\textsuperscript{61+} T cells have been previously reported to be inefficient killers of primary leukemia or lymphoma cells,\textsuperscript{44,45} which has been attributed to their lack of expression of NKG2D ligands.\textsuperscript{44} Interestingly, a recent study in which PBLs were activated with concanavalin A demonstrated higher killing ability of expanded V\textsuperscript{61+} cells against B-CLL–derived cell lines.\textsuperscript{47} It will be important to investigate whether this protocol also induces NKp30 expression on V\textsuperscript{61+} cells. Thus, NKp30\textsuperscript{+} V\textsuperscript{61+} cells may provide a valuable layer of intervention against lymphoid malignancies.

A surprising finding that deserves further investigation is the preferential expansion of V\textsuperscript{61+} T cells (among γδ PBL) on PHA treatment in vitro (Figure 3B). Because this is not due to selective apoptosis of the dominant V\textsuperscript{62+} counterparts (supplemental Figure 2), it must derive from a proliferative advantage of V\textsuperscript{61+} cells when receiving PHA-dependent TCR signals (Figure 3A-B). Provocatively, we have previously observed that V\textsuperscript{61+} T cells express significantly higher levels of the CD27 receptor (compared with V\textsuperscript{62+} cells)\textsuperscript{48}; CD27 costimulation enhances Bcl2a1 and Cyclin D2 expression and promotes T-cell survival and proliferation.\textsuperscript{48}

This study draws important novel (NCR-mediated) insight into the modus operandis of human T cells, and supports the emerging paradigm of T cells recognizing tumors via innate NK receptors rather than using the somatically rearranged TCR (Figure 5B-C).\textsuperscript{6} This notwithstanding, the TCR has a major, albeit indirect, contribution to the antitumor function of NKp30\textsuperscript{+} V\textsuperscript{61+} cells. We showed that efficient induction of NKp30 expression on V\textsuperscript{61+} cells depends on TCR stimulation; in its absence, γc cytokines can only effect a very modest up-regulation of NKp30 expression (Figure 4A-B). Thus, TCR signals are upstream of...
Nkp30-mediated tumor cell recognition by Nkp30+ Vδ1+ lymphocytes. Interestingly, we have previously shown that, for Vδ2+ cells, TCR signals are essential for cell activation and cytotoxic differentiation, "upstream" of tumor cell recognition via NKG2D.2 We therefore propose a "2-step" model for human γδ T cells, in which they differentiate and are activated like prototypic T cells (ie, using the TCR), but rely essentially on NK receptors (such as NKG2D, Nkp30 or DNAM-1) for tumor cell recognition. This is consistent with the critical role described by the Hayday and Girardi groups for NKG2D ligands in tumor surveillance by mouse γδ T cells,49,50 and fits the general concept of NK receptors being the key molecular recognition determinants of "oncogenic stress."2

From a clinical perspective, this study describes a protocol to induce Nkp30 ex vivo, which should make it very feasible to expand and inject large numbers of cells into patients. Importantly, we were able to efficiently expand Nkp30+ Vδ1+ cells from B-CLL patients (supplemental Figure 10). On reinfusion, the activation status of the cells could potentially be maintained via administration of low doses of IL-2, which appears to be sufficient to sustain Nkp30 expression. Thus, after this first report describing the differentiation of Nkp30+ Vδ1+ lymphocytes, future work should evaluate their potential for adoptive cell immunotherapy of human cancer.

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Authorship

Contribution: D.V.C., M.F., and K.H. performed the experiments and analyzed the data; M.G.S. provided vital materials and technical help; D.M. and B.S.-S. designed the study and analyzed the data; and B.S.-S. wrote the manuscript.

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