Synthetic Phosphoantigens Enhance Human $\gamma\delta V82$ T Lymphocytes Killing of Non-Hodgkin’s B Lymphoma

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Abstract

**Background:** Non-Hodgkin’s B lymphomas (NHL) are often resistant to conventional treatments and, until now, immunotherapeutic approaches against NHL only aimed at inducing $\alpha\beta$ anti-tumor effectors. Nevertheless, human blood $V82$ T lymphocytes represent an abundant pool of cytotoxic tumor-reactive cells. $V82$ T cells are strongly activated by natural compounds, from which powerful synthetic ligands have been derived. These synthetic antigens induce efficient $V82$ T cell responses in vitro.

**Materials and Methods:** We set up a series of $V82$ cell-activation experiments, including cytotoxic activity and amplification from whole blood cells. Several types of $V82$ effectors were challenged against a panel of 16 B lymphoma cell lines. These tests have been performed in the absence and presence of $\gamma\delta$-specific synthetic ligands to evaluate the effect of such molecules on $\gamma\delta$ anti-tumor activity.

**Results:** We report here that $V82$ T cells recognize B lymphomas. This recognition is associated with the cytotoxic activity against B-lymphoma cells and/or proliferative responses, and appears to be T-cell antigen receptor (TCR)-dependent. Because few B lymphoma induce a complete set of $V82$ cell responses, a chemical ligand of $V82$ T cells was used to enhance both proliferation and cytotoxic activity of anti-B lymphoma effectors. We show that such synthetic compound improves $V82$ CTL numbers and lysis of B lymphoma lines, especially when the targets are already spontaneously recognized by these effectors.

**Conclusions:** We report here that human $V82$ T cells anti-B lymphoma response can be improved by use of specific synthetic ligands, which enhance their cytotoxic activity and allows their rapid expansion ex vivo.

Introduction

Non-Hodgkin lymphomas (NHL) are lymphoproliferative disorders developing from B, T, or, rarely, natural killer (NK) cells. B cell NHL arise from the clonal expansion of a B cell developmentally blocked at virtually any stage of maturation (1). Increasing evidence suggests that a significant proportion of NHL B cells remain resistant to conventional chemotherapy (2–7). Despite their frequent infiltration by CD4$^+$ and CD8$^+$ T cells, B cell NHL rarely induce clinically significant T-cell-mediated responses (8–10). Clinical data suggest that it is partly due to the low frequency of tumor-infiltrating lymphocytes (TILs) and to their insufficient activated state in vivo [for a recent review see Schultz (11)]. Hence, autologous cytotoxic anti–NHL-specific T lymphocytes can be generated and expanded in vitro solely under very specific conditions, requiring cytokine-enriched media (12,13). However, such effectors do not always acquire significant anti-tumor cytotoxic activity (14). Because use of animal models has demonstrated the essential role of T cells in tumor rejection, most recent immunotherapeutic approaches aim at improving the in vivo activation of cytotoxic CD8$^+$ T lymphocytes (CTL) [for a review see Schultze and Nadler (15)]. Vaccination with B-cell NHL-associated isotype (the tumor’s most specific antigenic determinant) is one of the most studied strategies so far (16–20). Unfortunately, despite significant improvement, this approach still often shows uneven and unconvincing clinical efficacy (21).

Therefore, there is an obvious need for characterizing CTL populations with anti-tumor activity against B-cell NHL, as well as for defining simple approaches to amplify such anti-NHL–specific effectors.

In healthy human adult blood, around 3% of T cells express a $\gamma\delta$ T cell receptor (TCR), the vast majority of which is of the $V82$ subtype [for a review see De Libero (22)]. $V82$ T lymphocytes are known to accumulate preferentially at the sites of bacterial and parasitic infections (23–28) and are involved in anti-tumor control (29–34). On one hand, in infectious contexts, $V82$ T cells ligands are small protease-resistant phosphorylated molecules, termed “phosphoantigens” (27,35–37). Knowledge of the phosphoantigenic reactivity of $V82$ T cells has significantly improved in recent years [for recent reviews see Halary et al. (38), Belmant et al. (39), and Morita et al. (40)] and aided in the development of powerful synthetic phosphoantigens (41). On the other hand, $V82$ T lymphocytes exert two types of anti-tumor
activity. First, the broad antigen-specific recognition of hematopoietic tumors by Vγ9Vδ2 T lymphocytes results in cytotoxic activity, inducing Th1 cytokine production and proliferation (32,42). Classical Vγ9Vδ2-specific targets are the plasmacytoma RPMI8226 (43) and the Burkitt’s lymphoma Daudi (29,44–46), but so far, few if any other B-cell NHL have been described as targets of these CTL. Second, like NK cells, Vγ9Vδ2 T lymphocytes exert a cytotoxic activity controlled at the effector level by expression of killer Ig-like receptors (KIR) [for a review see Moretta et al. (47)], which interact with major histocompatibility complex (MHC) class I molecules at the surface of the target (32,48,49). Hence, tumor cell lines lacking expression of MHC-class I molecules, like chronic myelogenous leukemia K562 (29,45,50,51) or Burkitt’s lymphoma Daudi (45,48), are sensitive to this NK-like cytolytic activity. Despite such promising features, whether human Vγ9Vδ2 CTL act as effectors of an anti-tumor response against B-cell NHL is unknown.

In this study, we questioned the ability of synthetic phosphoantigens to improve the anti-B lymphoma activity of Vγ9Vδ2 T effectors. We provide evidence that γδ-specific synthetic ligands could constitute an efficient and convenient tool to enhance the anti-B lymphoma response of human Vγ9Vδ2 T cells to be tested in future immunotherapeutic approaches.

Materials and Methods

Tumor Cell Lines

All tumor cell lines were grown in Iscove’s Modified Dulbecco’s Medium (Biochrom KG, Berlin, Germany) supplemented with 100 U/ml penicillin/streptomycin, 2 mM glutamine, and 1 mM Na-pyruvate (complete medium), plus 20% heat-inactivated certified fetal calf serum (FCS) (Life Technologies, Paisley, Scotland), except Daudi, K562, RPMI8226, Jurkat, Raji, BL9, HLY-1, and RECl, which were grown in RPMI 1640–glutamax-1 (Life Technologies) medium supplemented with 100 U/ml penicillin/streptomycin and 1 mM Na-pyruvate plus 20% certified FCS (Life Technologies) and cell line DG75, which was grown in Dulbecco’s MEM [glutamax-1 medium supplemented with 100 U/ml penicillin/streptomycin and 1 mM Na-pyruvate plus 20% certified FCS (Life Technologies)]. Important features of each cell line of this study are listed in Table 1.

Purification of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were prepared from blood from healthy volunteers by centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.

### Table 1. Summary of characteristics of the tumor B-Cell lines involved in this study

| Maturation Stage | Cell Lines | Main Cytogenetic Abnormalities | EBV Status | Description | Reference |
|------------------|------------|-------------------------------|------------|-------------|-----------|
| pre-GC           | REC1       | t(11;14)                       | —          | Mantle cell NHL | (87)     |
|                  | OCI-Ly8    | t(14;18)                       | —          | DLC (CB/LB) NHL | (88)     |
|                  | DEAU       |                               | —          | DLC (CB) NHL   | (89)     |
|                  | VAL        | t(8;14;18) & t(3;4)            | —          | DLC (CB) NHL   | (90)     |
|                  | LIB        |                               | —          | DLC (IB) NHL   | *         |
| post-GC          | HLY-1      | t(3;5)                         | —          | (IB) NHL      | (91)     |
|                  | RL         | t(14;18)                       | —          | DLC NHL       | (92)     |
|                  | MIEUL      |                               | —          | Burkitt’s like NHL | *         |
| post-GC          | DAUDI      | t(8;14)                        | +          | Burkitt’s NHL | ATCC#CCL-213 |
|                  | RAJI       | t(8;14)                        | +          | Burkitt’s NHL | ATCC#CCL-86 |
|                  | BL9        |                               | +          | Burkitt’s NHL | Gift from G. Lyon, France |
|                  | DG75       | t(8;14)                        | —          | Burkitt’s NHL | (93)     |
|                  | PASC       | t(8;14)                        | —          | Burkitt’s NHL | *         |
|                  | L-428      |                               | —          | Hodgkin’s disease derived B cell, IgS~ | (94)     |
| post-GC          | NCI-H 929  |                               | —          | Plasmacytoma, IgA-producing | ATCC#CRL-9068 |
|                  | RPMI 8226  |                               | —          | Plasmacytoma, λ light chain-secreting | ATCC#CCL-155 |
| (non-B)          | K562       |                               | —          | Chronic myelogenous leukemia | ATCC#CCL-243 |
| (non-B)          | JURKAT     |                               | —          | T-lymphoblastic lymphoma/leukemia | ATCC#CRL-8163 |

*Cell lines established in our laboratory.
DLC, diffuse large cell; CB, centroblast; LB, lymphoblastic; IB, immunoblastic.
Generation of Vγ9Vδ2 T Lymphocyte Polyclonal Cell Lines

PBMC were added, at a final density of 1 to 2.10^6 cells/ml, to RPMI 1640–glutamax-1, 25 mM Hepes supplemented with 10 U/ml penicillin/streptomycin and 1 mM Na-pyruvate plus 10% heat-inactivated AB human serum (HS), with 100 U/ml recombinant human IL-2 (Sanofi-Synthelabo, Toulouse, France) and purified mycobacterial phosphoantigen 3-formyl-1-butyl pyrophosphate (3fβPP) (final concentration 5 nM). IL-2 was added every 5 days from day 5 at 50 U/ml final concentration. Between days 15 and 20, cell populations routinely reach over 95% Vγ9^+Vδ2^+ CD3^+ cells and can be either stored frozen or used as freshly derived polyclonal cell line.

Cell-Mediated Cytotoxic Assay

Vγ9Vδ2 T cells or PBMC cytotoxic activity was measured by standard 4-hr 51Cr (Na-bichromate, PHD, Innate Pharma, Marseilles, France) release assays in U-bottom 96-well microtiter plates in complete RPMI 1640 plus 5% heat-inactivated HS. Briefly, 3.10^5 51Cr-labeled targets were mixed with 6.10^4 (Vγ9Vδ2) or 3.10^5 (PBMC) effectors (final volume: 100 μl). When necessary, antibodies [antagonist anti-CD95, ZB4; anti-delta2, immu389; anti-gamma 9, immu360; anti-CD4, 13B8.2; anti-CD8, B9.11; isotype control mouse (m) IgG2a, 679.1Mc7, Immunotech-Beckman-Coupetu, Roissy, France] or agents [EGTA, tetrasodium salt, Sigma, St. Louis, MO, USA; PHD (formerly BrHPP, (41), Innate Pharma, Marseilles, France)] were added, at the indicated final concentrations, in supplementary 50 μl medium. Lysis of Jurkat cells by agonist anti-CD95 mAb (CH11; isotype control mlgM: GC32, Immunotech) was performed without γδ effectors. Maximum and spontaneous releases (MR and SR, respectively) were measured after incubation of the targets in medium alone, with half the labeled targets or half the supernatant, respectively. Percent specific lysis is given by (experimental release – SR)/(MR – SR). SR never exceeded 25% MR. For antibody-blocking experiments, target cells were incubated in HS during labeling to prevent antibody cross-linking by Fc-receptors. When needed, frozen polyclonal Vγ9Vδ2 T-cell lines were used as effectors immediately after thawing.

Induction of Surface CD69 Expression

Freshly prepared Vγ9Vδ2 T cells (10^6; day 17 after 3fβPP amplification) were mixed with BCECF-stained and washed tumor cells in 200 μl complete RPMI 1640 plus 10% HS, with a γδ/target ratio of 1/5. As positive control, Phorbol 12-Myristate 13-Acetate (PMA, Sigma) was added at 1 μg/ml. After 8-hr incubation, cells were washed in phosphate-buffered saline (PBS) plus 0.5mM EDTA and stained with PE-conjugated CD69 monoconal antibody (mAb) (TP1.55.3, Immunotech), after gating on viable, BCECF unstained cells.

In Vitro Amplification of Vγ9Vδ2 T Cells From PBMC

PBMC (5–10–10^5) were cultured in 48-well microtiter plates, in 1-ml complete RPMI 1640, 100 U/ml recombinant human IL-2 (Sanofi-Synthelabo) plus 10% heat-inactivated AB HS in the presence of 3fβPP (5nM), various final concentrations of PHD (Innate Pharma, Marseilles, France) (as indicated), or with 2.5.10^5 Mitomycin C-treated (Sigma) and washed tumor cell targets. Fifty to 100 U/ml IL-2 were added at days 5 and 10, and amplification of Vγ9Vδ2 T cells was measured by fluorescence activated cell sorting (FACS) analysis. Increase in Vγ9Vδ2 T-cell numbers was calculated as: [% δ2^+CD3^+ cells after stimulation (AS) x total viable cell number AS] / [% δ2^+CD3^+ cells before stimulation (BS) x total viable cell number BS].

FACS Analysis

HLA class I surface expression on tumor targets induced CD69 surface expression on Vγ9Vδ2 CTL and Vγ9Vδ2 amplification from PBMC were monitored by one- or two-color FACS analysis. Anti-HLA class I mAb W6/32 staining was revealed with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (GAM) mAb (Immunotech). FITC-conjugated anti-Vδ2 and phycoerythrin (PE)-conjugated anti-CD3 mAbs (immu389 and UCHT1, Immunotech) stain Vγ9Vδ2 T cells. PE-conjugated CD69 mAb (TP1.55.3, Immunotech) stains activated Vγ9Vδ2 T cells. The following isotype-matched antibodies were used as controls: mouse (m) IgG2a, U7.27; mlgG1-FITC, mlgG1-PE, 679.1Mc7; mlgG2b-RD1, MOPC-195 (Immunotech). Analyses were performed after gating on viable cells on a Beckman-Coulter apparatus.

Results

Polyclonal Vγ9Vδ2 CTL Kill B Cell NHL Lines In Vitro

The in vitro cytotoxicity of two unrelated primary Vγ9Vδ2 cell lines derived from two different healthy donors was evaluated against 15 non-Hodgkin's B lymphoma cell lines and one Hodgkin's disease-derived B-cell lymphoma line. These B-cell lymphomas were selected such as to represent various stages of B-cell differentiation (see Table 1).

The percentages of specific lysis of each polyclonal Vγ9Vδ2 cells against the panel of NHL are presented in Figure 1. Although overall cytotoxicity of the first polyclonal Vγ9Vδ2 CTL line is reproducibly lower than that of the second, both polyclonal CTL lines exert comparable levels of cytotoxic response against each individual target. For any tested CTL line, the level of spontaneous cytotoxicity is heterogeneous with regard to the target: seven NHL lines are not spontaneously lysed by Vγ9Vδ2 CTL in vitro (below 10% specific lysis, see Fig. 1, bottom). Six NHL cell lines spontaneously trigger intermediate levels of killing by Vγ9Vδ2 cells (10–40% specific lysis: PASC, HLY-1, BL9, RPM18226, VAL, and DG75) and two NHL lines and the Hodgkin’s lymphoma line
spontaneously activate a high level (above 40% of specific lysis; Fig. 1, top) of specific lysis by γδ effectors.

Cytolytic activity of Vγ9Vδ2 CTL for tumor cells is known to be influenced by a deficit in HLA class I molecule expression at the surface of the targets (32,48,49,52), accounting for an NK-like γδ-mediated killing of the HLA-class I− targets K562 and Daudi (32,45,50,51). To rule out such a possibility, we evaluated the level of surface expression of HLA class I molecules on the target lymphoma lines with the W6/32 mAb directed against HLA-A, -B, -C, -E, and -G (Fig. 1). Whereas NHL lines differ in terms of intensity of HLA class I expression, Figure 1 shows that the specific lysis of these targets by Vγ9Vδ2 CTL does not match to their relative deficit in surface expression of HLA class I molecules. The HLA-class Ilow lymphoma REC1 and PASC are not killed, whereas several HLA-class I bright lines (OCI-Ly8, DG75, RPMI8226, and VAL) are efficiently lysed (Fig. 1). Thus, in contrast to K562 and Daudi cell lines, the OCI-Ly8, VAL, DG75, BL9, RPMI8226, and HLY-1 NHL are killed by Vγ9Vδ2 CTL solely through a non-NK-like pathway.

Furthermore, Vγ9Vδ2 T cells spontaneous cytotoxicity toward these NHL B cell lines is not specifically restricted to Epstein-Barr Virus (EBV)-positive lymphomas (Fig. 1).

**Amplification of Vγ9Vδ2 T Cells in Response to B-Cell NHL Lines**

Vγ9Vδ2 CTL lymphocytes proliferate in vitro when grown in the presence of some hematopoietic neoplastic cell lines (32), such as the Burkitt's lymphoma Daudi (29,45) and the plasmacytoma RPMI8226 (43). However, Vγ9Vδ2 CTL do not proliferate in vitro when grown with their targets of NK-like lysis [e.g., the chronic myelogenous leukemia K562 (32,45)].

Because several B cell lymphoma lines from the above panel activate γδ-selective cytotoxicity, we asked whether these irradiated lymphoma could also induce a selective outgrowth of polyclonal Vγ9Vδ2 T lymphocytes in vitro when co-cultured with freshly purified PBMC from several healthy donors. As expected, Daudi and RPMI8226 cell lines induce expansion of Vγ9Vδ2 T cells from PBMC (Table 2). Similarly, Hodgkin's lymphoma line L428 triggers both high Vγ9Vδ2 lysis and Vγ9Vδ2 CTL expansion from PBMC of two on three donors (Table 2). Interestingly, the NHL line DEAU, which does not induce γδ cytotoxicity, promotes Vγ9Vδ2 T cell outgrowth from PBMC in two of three donors. None of the other NHL lines tested expand Vγ9Vδ2 CTL from any PBMC tested, although some of them, like OCI-Ly8, DG75, and VAL, trigger strong Vγ9Vδ2 T cell cytotoxic activity. Thus, of several NHL B-cell lines activating Vγ9Vδ2 T cells, few induce both cytotoxicity and amplification responses.

B lymphoma cell lines involved in this study were chosen such as to stretch along the whole B-cell differentiation process (Table 1). This panel comprises B-cell malignancies starting from pregerminal centers (pre-GC) cells, GC/post-GC cells up to immunoglobulin (Ig)-producing cells. Looking for a possible correlate between γδ-activating phenotype and B-cell differentiation, we compared the γδ-stimulating properties of these 16 B lymphoma and their respective stage of differentiation. This comparison suggested that γδ-activating B malignancies span all along B-cell maturation.

**Specificity of Spontaneous Vγ9Vδ2 CTL Cytotoxicity to NHL B-Cell Lines**

The unusual pattern of Vγ9Vδ2 CTL responses when exposed to NHL B-cell lines questioned the nature of the Vγ9Vδ2 activation pathway by NHL. Using OCI-Ly8 as a model of Vγ9Vδ2 CTL-activating NHL, we investigated some characteristics of its killing pathway and the involvement of the Vγ9Vδ2 TCR. As shown in Figure 1, the HLA-class I bright OCI-Ly8 NHL is efficiently and spontaneously killed by Vγ9Vδ2 T lymphocytes in vitro but fails to induce Vγ9Vδ2 CTL expansion from primary PBMC cultures.
Although Fas-mediated cytotoxicity is assumed to be negligible in 4-hr chromium release assay (53,54), we asked whether the strong lysis of OCI-Ly8 by Vγ9Vδ2 CTL relies on a marked sensitivity of this NHL line to Fas-L. For this purpose, OCI-Ly8 killing by Vγ9Vδ2 CTL was tested in presence of Fas-agonist CH11 mAb (55), which induces the apoptotic death of Fas−/Jurkat cells (Fig. 2A), or conversely in presence of the Fas-antagonist ZB4 mAb (56), which inhibits CH11-induced apoptosis (Fig. 2A). In these experiments, killing of Fas-Daudi or of OCI-Ly8 by Vγ9Vδ2 T lymphocytes was not altered by the Fas antagonist (Fig. 2A).

Because TCR-mediated activation of CTL usually leads to perforin release [for a review see Shresta et al. (57)], we tested whether OCI-Ly8 line killing involves the release of perforin by Vγ9Vδ2 T cells. Although it does not alter the Fas-mediated T-cell cytotoxicity (58), EGTA inhibits the calcium-dependent release of perforin (and other soluble mediators) from exocytosis granules. When added to the in vitro mix of OCI-Ly8 and Vγ9Vδ2 CTL, EGTA totally suppresses killing of OCI-Ly8 by Vγ9Vδ2 T cells (Fig. 2B). In contrast to earlier reports (59,60), in our experiments Vγ9Vδ2 T cells kill Jurkat targets in a strictly Ca2+-dependent way (Fig. 2B). EGTA has, however, no effect on the lysis of Jurkat cells induced by the Fas-agonist (CH11 mAb). Isotype-matched IgG1 antibody has no effect on CH11-induced lysis of Jurkat cells (not shown). ZB4 mAb concentrations are shown in insert box. NT, not tested; E/T = 20/1. (B) Effect of Ca2+-chelating agent EGTA on Vγ9Vδ2 T-cell cytotoxicity toward Daudi, OCI-Ly8, and Jurkat cells in a 4-hr 51chromium release assay. As a negative control, EGTA effect was also tested on Jurkat cells lysis induced by Fas agonist CH11 mAb in the same conditions. Isotype-matched IgM antibody induced no lysis of Jurkat cells (not shown). EGTA concentrations are shown in insert box. (E/T = 20/1).

Table 2. Amplification of Vγ9Vδ2 T cells after incubation with various NHL B-cell lymphoma

| Cell Lines | Vγ9Vδ2 CTL Expansion from PBMC/Number of Donors Tested |
|------------|--------------------------------------------------------|
| DAUDI      | 3/3                                                    |
| OCI-Ly8    | 0/3                                                    |
| L428       | 2/3                                                    |
| DG75       | 0/2                                                    |
| VAL        | 0/3                                                    |
| RPMI8226   | 3/3                                                    |
| BL9        | 0/2                                                    |
| HLY-1      | 0/2                                                    |
| PASC       | 0/2                                                    |
| DEAU       | 2/3                                                    |
| NCI-H 929  | 0/2                                                    |
| RL         | 0/3                                                    |
| LIB        | 0/2                                                    |
| REC1       | 0/2                                                    |
| MIEUL      | 0/2                                                    |
| RAJI       | 0/3                                                    |

After 13-day co-culture of primary PBMC from healthy donors with irradiated NHL-cells, viable CD3+ TCR-Vδ2+ cells were quantified by FACS. Amplification was considered negative when percentage of CD3+ TCRVδ2+ cells in co-cultures was similar to that of PBMC cultured with IL-2 only. For each NHL, data summarize the ratio of amplification-reactive donors versus number of donors tested. For example, with the stimulating NHL DEAU, representative percentages of CD3+ TCRVδ2+ cells are: donor 1, 9.9% in co-culture versus 3.1% for IL-2 alone; donor 2, 3.8% in co-culture versus 2.4% for IL-2 alone; donor 3, 18.1% in co-culture versus 2.8% for IL-2 alone. With nonstimulating NHL DG75: representative percentages of CD3+ TCRVδ2+ cells are donor 1, 2.1% in co-culture versus 3.1% for IL-2 alone; donor 2, 1.1% in co-culture versus 2.4% for IL-2 alone.

To address this point more directly, OCI-Ly8 and Vγ9Vδ2 CTL were co-incubated in the presence of increasing quantities of the anti-TCR Vδ2 mAb Immu389 (61). Figure 3 shows that this
antibody strongly blocks lysis of OCI-Ly8 as well as the target RPMI8226 by polyclonal Vγ9Vδ2 CTL. The anti-TCR Vδ2 mAb only partially inhibits lysis of HLA-class I− Daudi lymphoma, which results both from TCR-mediated and NK-like lysis by Vγ9Vδ2 T cells. As reported (49,50), the anti-TCR Vδ2 mAb Immu389 does not inhibit lysis of HLA-class I− K562 tumor cells (Fig. 3A). In line with these results, while mAb directed against TCR Vγ9 chains reduces the lysis of OCI-Ly8 by Vγ9Vδ2 T cells, mAbs against CD4 and CD8 seldom expressed on polyclonal cells have no effect on this lysis (Fig. 3B). Taken together, these results support the idea that the cytotoxicity of Vγ9Vδ2 CTL for NHL target OCI-Ly8 is mediated by the γδ2-TCR.

Upon antigen recognition, the TCR mediates initial steps of T-cell activation, ultimately followed by functional T-cell responses. Because γδ2-TCR-mediated recognition of OCI-Ly8 NHL drives further cytotoxic responses, we asked whether NHL stimulation of Vγ9Vδ2 CTL induces early appearance of activation markers on these effectors. Antigenic activation of T cells induces the surface expression of specific markers, of which CD69 is one of the earliest [for recent reviews, see Tough 2t al. (62) and Marzio et al. (63)]. Thus, following exposure to different tumor cell lines, we tested the induction of CD69 at the surface of freshly derived Vγ9Vδ2 T lymphocytes. As compared to unstimulated γδ cells alone (negative control, Fig. 4) and PMA-treated γδ cells (positive control, Fig. 4), Daudi, OCI-Ly8 NHL, or RPMI8226 activate CD69 expression (Fig. 4). Conversely, neither of the γδ unstimulatory Jurkat and K562 tumor cells do so (Fig. 4). In these experiments, NHL line OCI-Ly8 induces clear-cut expression of the activation marker CD69 at the surface of polyclonal Vγ9Vδ2 T lymphocytes as early as 8 hr after co-culture, witnessing early induction by a Vγ9Vδ2 TCR-mediated activation (Fig. 4).

Taken together, these results demonstrate that although they do not necessarily induce γδ T-cell proliferation, B lymphoma lines may be specifically

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**Fig. 3.** TCR requirement for Vγ9Vδ2 cytotoxicity toward OCI-Ly8. (A) Effect of increasing concentrations of immu389 anti-delta2 mAb on Vγ9Vδ2 T-cell cytotoxicity toward Daudi, RPMI8226, K562, and OCI-Ly8. Isotypic IgG1 (at 10 μg/ml) antibody was used as a negative control. Immu389 mAb dilutions are shown in insert box. (E/T = 20/1). (B) Effect of diverse antibodies against CTL surface antigens on polyclonal Vγ9Vδ2 T-cell line cytotoxicity toward the follicular lymphoma cell line OCI-Ly8. The four mAb tested share the same isotype (IgG1), shown as negative control. Antibodies dilutions are shown in insert box. (E/T = 20/1).

**Fig. 4.** Expression of the early activation surface marker CD69 by polyclonal Vγ9Vδ2 T-cell line after exposure to different cells. A freshly derived polyclonal Vγ9Vδ2 T cells population was exposed to several tumor cells in vitro for 8 hr. CD69-surface expression on Vγ9Vδ2 T cells with IgG2b-RD1 isotype antibody (gray line) and with PE-conjugated anti-CD69 antibody after incubation with medium alone.
recognized by Vγ9Vδ2 TCR and consequently be killed by these effector cells.

Drug-Induced Amplification of Vγ9Vδ2 CTL Cell Numbers and Anti-NHL Cytotoxicity

Because γδ-stimulating NHL B-cell lines fail to induce a complete set of Vγ9Vδ2 CTL responses in vitro (Fig. 1 and Table 2), this limits the clinical potential of spontaneous activated γδ cells. However, novel drugs have been recently designed that trigger the complete set of Vγ9Vδ2 T cell responses in vitro (41). This prompted us to test whether such synthetic drugs could circumvent the absence of expansion of these γδ effectors while maintaining (or enhancing) their anti-NHL cytotoxicity.

To compensate for the absence of amplification of Vγ9Vδ2 effectors in response to target B lymphomas (Table 2), we used synthetic drugs to generate high quantities of anti-B lymphoma Vγ9Vδ2 CTL in vitro. These drugs (39,41) specifically mimic natural Vγ9Vδ2-T-cell ligands [referred to as phosphoantigens (27,35–37,64,65)]. Increasing concentrations of the drug PHD (41) were added to primary PBMC cultures from four healthy donors (Fig. 5). There was a strong increase in Vγ9Vδ2 T-cell numbers from PBMC of all donors, reaching more than 30-fold (Fig. 5A). This amplification is dose dependent, as shown for donor 1 (Fig. 5B). Finally, Figure 5C shows the maximal percentage of Vγ9Vδ2 T cells obtained for the three other donors.

Thus, although B-lymphoma lines as stimuli may sometimes fail to amplify specific CTL in vitro, these effectors can nevertheless be conveniently amplified in vitro using synthetic drugs. Because some NHL B-cell lines appear resistant to Vγ9Vδ2 CTL lysis in vitro, we also investigated the ability of PHD to improve the cytolytic activity of γδ effectors. Here, we analyzed the effect of this drug on the cytotoxic activity of two Vγ9Vδ2 T-cell lines against the panel of B lymphomas. A final concentration of 20 nM PHD was added in these cytotoxic assays, where the effector-to-target (E/T) ratio decreases from 20/1 to 2.5/1. Figure 6 shows the effect of PHD added to the anti-NHL cytotoxicity of Vγ9Vδ2 CTL. PHD effect on NHL killing by γδ effectors is heterogeneous, as observed above for their spontaneous anti-NHL cytotoxicity. On the one hand, PHD improves slightly the killing of genuine γδ-activating targets such as Daudi, OCI-Ly8, L428, DG754, or VAL (Fig. 6). On the other hand, PHD does not elicit killing of unstimulatory NHL lines (Raji, MIEUL, LIB, or REC1, Fig. 6). Interestingly, PHD increases by about 10-fold the anti-NHL cytolytic potential of Vγ9Vδ2 CTL against some targets (see lysis of HLY-1, NCI-H 929, and RL in Fig. 6). PHD appears most efficient in enhancing Vγ9Vδ2 T-cell cytotoxicity toward partially activating NHL B-cell lines.

Because the PHD-induced increase in γδ anti-NHL activity had only been challenged on long-term

preactivated γδ primary cell lines, we questioned the relevance of such a bioactivity for B lymphoma targets within freshly drawn γδ T cells. Thus, the effect of PHD stimulation on anti-NHL Vγ9Vδ2 fresh CTL was tested by setting cytotoxicity experiments with freshly prepared PBMC effectors. Primary PBMC from two healthy donors with distinct Vγ9Vδ2 T-cell proportions were tested extemporaneously in a 4-hr chromium release assay, for their
lysis of the preceding panel of human B lymphomas (Fig. 7). As already observed with long-term polyclonal γδ-cell lines (see Fig. 1), freshly prepared PBMC from the two different donors spontaneously exert anti-NHL cytotoxicity, although of different intensity according to the target. Adding PHD (final concentration 200 and 800 nM, without IL-2) to such CTL assays gave roughly a similar pattern of effect as formerly observed using PHD-amplified γδ effectors (Fig. 7). Killing of genuine B lymphoma targets and of stimulating NHL is significantly improved, with a PHD dose effect (see Daudi, OCI-Ly8, L428, DG75, VAL, BL9, Fig. 7), while resistant NHL remain unaffected (see HLY-1, DEAU, MIEUL and Raji, Fig. 7). In these experiments, the PHD effect was not greatly influenced by the ratio of PHD-reactive Vγ9Vδ2 T cells among PBMC (e.g., the VAL NHL line is killed similarly by PHD-treated PBMC from donor 1 and donor 2, Fig. 7). Furthermore, PHD exposure of freshly prepared PBMC induces a low level of killing of some otherwise resistant cell lines (see NCI-H 929, RL or LIB, Fig. 7).

Discussion
This in vitro study aimed at documenting the cytotoxic potential of human Vγ9Vδ2 T lymphocytes against non-Hodgkin's B-cell lymphoma lines and the ability of γδ synthetic ligands to improve this anti-B lymphoma activity. We show that this CTL population is spontaneously activated to kill several NHL B-cell lines in vitro. As judged by HLA class I molecule expression at the surface of the targets, this cytotoxicity does not result from an NK-like lysis, but most probably arises from a specific TCR-mediated stimulation. The features of the killing mechanism of the target NHL line OCI-Ly8 by Vγ9Vδ2 CTL confirms their specific activation. Thus, human Vγ9Vδ2 T cells represent potential anti-NHL CTL, of high frequency among circulating T cells in blood of healthy donors (approximately 1–10%) (22).

Nevertheless, the activation of Vγ9Vδ2 CTL by B lymphoma cell lines does not necessarily lead to a...
complete set of T-cell activation phenotypes. The NHL DEAU cell line induces their specific amplification from primary PBMC while failing to activate Vγ9Vδ2 T-cell cytotoxicity. Furthermore, only three of nine B lymphoma activating Vγ9Vδ2 T-cell cytotoxicities also promote Vγ9Vδ2 CTL expansion. In this respect, it has often been reported that NHL cells hardly stimulate αβ CTL proliferation in vitro unless expression of several costimulatory molecules is induced at their surface (66–72).

Absence of Vγ9Vδ2 CTL amplification following contact with NHL can be conveniently overcome using drugs specific for the γδ-TCR. Anti-NHL cytotoxic Vγ9Vδ2 T lymphocytes expand upon stimulation with natural phosphoantigens such as 3βPP (35,64) or synthetic analogs such as PHD. This amplification requires IL-2 and very low concentrations of the drug PHD (5–50 nM) within 10 days. Moreover, these molecules can also significantly enhance the anti-NHL cytotoxic activity of both polyclonal Vγ9Vδ2 T-cell lines and freshly prepared Vγ9Vδ2 T cells within PBMC. The drug-induced improvement of anti-B lymphoma activity is particularly significant toward targets that otherwise spontaneously induce a low level of Vγ9Vδ2 T-cell cytotoxicity. Variable levels of basal B lymphoma lysis by PBMC CTL are observed within donors (Fig. 7). It is assumed that Vγ9Vδ2 T cells account for some of this initial cytotoxicity. However, PHD-dependent increase in B lymphoma lysis is solely mediated by responding Vγ9Vδ2 T cells; natural phosphoantigens and their synthetic counterparts do not stimulate other cellular effectors (35,41,73) (Fig. 5). Interestingly, PHD not only improves the lysis of γδ-sensitive B lymphoma lines, but also confers low cytotoxicity to Vγ9Vδ2 CTL toward usually resistant cell lines. Unfortunately, one third of the NHL lines tested are not killed, even by effectors stimulated with their specific ligand. We assume that intrinsic NHL-resistance to granzyme-perforin-mediated lysis could account for such resistance.

The panel of B-cell lymphoma involved in this study was chosen because it comprises malignant counterparts of B cells at various stages of differentiation (Table 1). Thus, we questioned the existence of a correlation between the γδ stimulation property and the level of B-cell target differentiation. However, because γδ-stimulating B cells span all along the different maturation steps, we could not link the Vγ9Vδ2 T-cell–stimulating property to the B-cell differentiation stage. This finding is in agreement with the assumed ubiquity of the B lymphoma ligands of Vγ9Vδ2 T cells (32), which remain unknown. Nevertheless, with regard to the nature of these antigens, this study suggests that Vγ9Vδ2 tumor antigens are most probably not related to (1) maturation-dependent B cell markers, (2) B-cell–activation specific molecules, or (3) B-cell receptor (BCR)-expression and Ig secretion. Thus, one may consider Vγ9Vδ2 CTL as potential effectors of almost any B malignancy.

Immunotherapeutic trials against NHL aim at eliciting specific cytotoxic T-cell responses against these cancer cells (21). Generally, the candidate effectors are αβ CD8+ T lymphocytes, because they can be manipulated by two distinct approaches. These effectors are either stimulated in vivo after vaccination with a tumor-specific antigenic determinant (16,17,19,20) or they are expanded ex vivo as CTL against the autologous tumor (12,13,69). The first approach has improved through monitoring the acquired immunity and a better detection of the residual disease. Nevertheless, vaccination still requires further improvement for its generalization in anti-lymphoma protection (21). More specifically, cancer vaccination would benefit from the identification of novel specific tumor antigens (74). Ex vivo generation of specific autologous CTL has proven difficult for the relatively low TILs frequency, their poor intrinsic cytotoxic activity, and their sophisticated culture conditions (12,13,69,75,76). Therefore, a need for the identification of novel effectors of the anti-lymphoma immune response still remains. This study supports new options for the design of anti-tumor cellular immunotherapies. As yet, however, no report describes autologous Vγ9Vδ2 T lymphocytes as NHL-TILs in vivo (77,78) or as CTL amplified in vitro from TILs (13); our study indicates that reactive Vγ9Vδ2 CTL against B lymphomas can be readily generated in vitro. In this context, human Vγ9Vδ2 T lymphocytes offer several advantages as cellular effectors as compared to αβ cytotoxic T cells. Above all, whereas the usual frequency in blood of almost any αβ CTL is below 0.01%, that of Vγ9Vδ2 CTL is quite higher, being 1–10% in adults (22). Furthermore, polyclonal Vγ9Vδ2 CTL cell lines expand within a few days after stimulation of PBMC with specific ligands (35,36,51) (Fig. 5). As effectors of innate immunity, Vγ9Vδ2 T lymphocytes acquire cytotoxic activity against tumor target without former exposure (79), whereas alloreactive CD8+ CTL have to be primed to become efficient responders to NHL cells (12,66,68,70). Furthermore, once generated from PBMC through phosphoantigenic stimulation, Vγ9Vδ2 T lymphocytes simultaneously acquire responsiveness to several distinct target cells (34,50,79) (this study). Thus, generating autologous activated γδ T cells is far simpler than expanding αβ CTL against NHL cells in the presence of the patient's tumor cells (12,13). Finally, the recent development of chemical ligands for Vγ9Vδ2 T cells even reinforces their interest for the design of future antilymphoma immunotherapeutic tests. The synthesis of chemical ligands is easier and less expensive than the purification of natural phosphoantigens from microbial sources. Moreover, for obvious safety reasons, the use of synthetic ligands is easier to control than that of extracts from pathogens such as M. tuberculosis.

Another advantage of the Vγ9Vδ2 CTL population is that its reactivity and functionality is not MHC restricted (29,41,44,79–81). On the other hand,
weak αβ T-cell response against B lymphoma is partly due to the poor antigen-presenting cell (APC) function of the tumor cells (66,70,82). Hence, V9Vδ2 CTL are interesting effectors because their cytotoxic response does not depend on a classical presentation of tumor antigens, also circumventing the need for improving ex vivo the APC function of dendritic cells with tumor antigens (83–86). In conclusion, this study suggests that the V9Vδ2 population of CTL should be considered as a potential pool of anti-NHL effectors, for which novel powerful stimulating drugs are now available.

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